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An examination of quality management in an investigation of mechanisms of 5-fluorouracil and gemcitabine resistance in colorectal and pancreatic cancers

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Aim at perfection in everything, though in most things it is unattainable. However, they who aim at it, and persevere, will come much nearer to it than those whose laziness and despondency make them give it up as unattainable.

Lord Chesterfield

Declaration

I hereby declare that this thesis was completed by the author. The research work is original according to the regulations of the University of Edinburgh. Any sources of information have been acknowledged. Any work not completed by the student has been acknowledged. This work has not been submitted for any other degree or award.

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Abstract

Recent publications have repeatedly highlighted the unreliability of academic research when translated to industry. This subject has been debated in publications such as Nature Drug Discovery, RQA monthly, Life Sci VC and Lab Times with many reasons cited including research bias, academic self-interest, apathy, pressure to publish and "*massaging data to fit the hypothesis*". [1 5]

Resistance in colon and pancreatic cancer is well documented but the literature is conflicted. Perhaps this is because of biological variability but it could be due to experimental design. The aim of this project is to conduct a study, mindful at each stage of how quality management might inform the interpretation of findings

Colorectal cancer is the third most common cancer for both males and females in the UK and makes up 13% of all cancers.[2] 5-fluorouracil (5-FU) has been, and remains, the first line drug of choice for treating colorectal cancer, both as a monotherapy and in combination with other drugs. However, the response rate is around 20% when used singly and just under 50% when used in combination.[3 11 113]

Pancreatic cancer is the tenth most common cancer in the UK and makes up 2.55% of all cancers. The average survival time for a pancreatic cancer patient following surgical resection is approximately 11-20 months. This drops to 6-11 months in unresectable localised tumour patients. For patients with unresectable, metastatic disease the average survival time is 2-6 months.

Although 5-FU and gemcitabine remain the first line treatment for colorectal and pancreatic cancers, there are no definitive markers of resistance which may be used to predict response to these drugs.

Investigation into prognostic and predictive biomarkers based upon putative resistance to these drugs is crucial to tailor therapy in patients with a potentially very limited lifespan. Automated quantitative analysis (AQUA) is a novel image analysis system of quantifying protein expression levels, including potential biomarkers, in cells.

This thesis addresses the hypothesis that quantitative expression of protein expression can be used to identify putative biomarkers of gemcitabine resistance in pancreatic cancer, and that the methodology is transferrable to other disease types. Using pancreatic cancer as the lead disease this project aims to measure the expression levels of proteins critical for both pathways – thymidylate synthetase (TS), ribonucleotide reductase subunit M1 (RRM1), ribonucleotide reductase subunit M2 (RRM2), cytidine deaminase (CDA), human equilibrative nucleoside transporter 1

(hENT1) - and pathway specific proteins – dihydropyrimidine dehydrogenase (DPYD) and thymidine phosphorylase (TP) for 5-FU- and a gemcitabine pathway-specific protein, deoxycytidine kinase (DCK). Thereafter, in a briefer study, the application of the approach to colorectal cancer was investigated.

Archival FFPE blocks were used to construct tissue microarrays (TMAs) in quadruplicate and patient demographics were collated. An antibody validation algorithm was formulated to authenticate antibodies prior to use. A quality programme was initiated and maintained throughout the length of the project to ensure data integrity and reproducibility of results. The expression levels of the proteins were quantified using Automated QUantitative Analysis (AQUA).

Low CDA and RRM1 expression were associated with longer disease free survival in all patients. Medium CDA expression was associated with longer overall survival in the patients who did not receive any chemotherapy. Low RRM1 expression was linked with longer overall and disease free survival in the gemcitabine cohort. Multivariate analysis showed that a high Lecca value was linked to increased survival time, statistically significant in the gemcitabine group – $p = 0.007$ for overall survival and $p = 0.07$ for disease free survival.

24% of patients who received no chemotherapy (high expression group) were still alive at 48 months and 25% of patients who received gemcitabine (high expression group) were still alive at 72 months.

In the low expression groups, both sets of patients had the same amount of time to disease progression, 14 months.

Lay Summary

Colorectal cancer is the third most common cancer for both males and females in the UK and makes up 13% of all cancers. 5-fluorouracil (5-FU) has been, and remains, the first line drug of choice for treating colorectal cancer, used on its own as well as in combination with other drugs. However, the response rate is around 20% when used singly and just under 50% when used with other drugs.

Pancreatic cancer is the tenth most common cancer in the UK and makes up 2.55% of all cancers. The average survival time for a pancreatic cancer patient following surgery to remove the tumour is approximately 11-20 months. This drops to 6-11 months in patients with tumour that is only located in the pancreas and cannot be removed by surgery. For patients with tumour that has spread to other parts of the body the average survival time is 2-6 months. Gemcitabine is the first line drug of choice to treat pancreatic cancer.

Resistance of colorectal and pancreatic cancer is well documented but reasons reported contradict one another. This could be due to variations in the tumours but also could be due to lack of quality control measures. This project was conducted with a quality programme implemented to try and work out what causes resistance or response to 5-FU and gemcitabine by looking at substances expressed or produced in the tumour cells which metabolise or activate 5-FU and gemcitabine.

Abbreviations

5-FU	5-Fluorouracil
AQUA	Automated QUantitative Analysis
BCA	Bicinchoninic Acid Assay
BMI	Body Mass Index
BRCA2	Breast Cancer 2
CA19-9	Cancer Antigen 19-9
CAPOX	CAPecitabine, OXaliplatin
CDA	Cytidine Deaminase
CEA	CarcinoEmbryonic Antigen
CPA	Clinical Pathology Accreditation (UK) Ltd.
CRUK	Cancer Research UK
CT	Computerised Tomography
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCK	Deoxycytidine Kinase
DFS	Disease Free Survival
DNA	Deoxyribonucleic Acid
DPYD	Dihydropyrimidine Dehydrogenase
ERCP	Endoscopic Retrograde Cholangio-Pancreatography
ERK	Extracellular signal-Regulated Kinases
ESPAC	European Study group for PAncreatic Cancer
EUROPAC	European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer
EUS	Endoluminal Ultrasound
FAMMM	Familial Atypical Multiple Mole Melanoma
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration (US)
FFPE	Formalin Fixed Paraffin Embedded
FISH	Fluorescent In-Situ Hybridisation
FNA	Fine Needle Aspiration
FOLFIRINOX	FOLinic acid, Fluorouracil, IRINotecan, OXaliplatin
FOLFOX	FOLinic acid, Fluorouracil, OXaliplatin
GCP	Good Clinical Practice

GCLP	Good Clinical Laboratory Practice
GEMCAP	GEMcitabine, CAPecitabine
GLP	Good Laboratory Practice
GP	General Practitioner
H&E	Haematoxylin and Eosin
hENT1	Human Equilibrative Nucleoside Transporter 1
HER2	Human Epidermal growth factor Receptor 2
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HPA	Human Protein Atlas
HPV	Human Papilloma Virus
HRT	Hormone Replacement Therapy
ICH	International Conference on Harmonisation
IF	Immunofluorescence
IHC	Immunohistochemistry
IPMN	Intraductal Papillary Mucinous Neoplasm
ISD	Information Services Division (Scotland)
LKB1	Liver Kinase B1
MAPK	Mitogen-Activated Protein Kinases
MRI	Magnetic Resonance Imaging
MW	Molecular Weight
NK	Not Known
OC	Oral Contraceptive
OECD	Organisation for Economic Cooperation and Development
OS	Overall Survival
PBS	Phosphate Buffered Saline
PDAC	Pancreatic Ductal AdenoCarcinoma
PI	Principal Investigator
PLACE	Pixel based Locale Assignment for Compartmentalisation of Expression
QA	Quality Assurance
QC	Quality Control
REC	Research Ethics Committee
RESA	Rapid Exponential Subtraction Algorithm
RNA	Ribonucleic Acid
RPPA	Reverse Phase Protein Arrays

RRM1	Ribonucleotide Reductase sub-unit M1
RRM2	Ribonucleotide Reductase sub-unit M2
RQA	Research Quality Assurance
SD	Standard Deviation
SI	Statutory Instrument
SOP	Standard Operating Procedure
SPSS	Statistical Package for the Social Sciences
STK11	Serine/Threonine Kinase 11
TAM	Tumour Associated Macrophage
TMA	Tissue Microarray
TNM	Tumour Node Metastases
TP	Thymidine Phosphorylase
TS	Thymidylate Synth(et)ase
WHO	World Health Organisation
XELOX	XELoda, OXaliplatin

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1. Introduction and background

1.1 The quality of academic research

There have been many recent reports on the quality, or lack of it, of academic research. The irreproducibility of academic research has been highlighted in many articles. [1] It has been reported that at least 50% of articles published in elite journals cannot be reproduced by other labs. [1 5] The reasons cited for this include research bias and academic self-interest. [5] Researchers have a clear vision of what they expect from their research and, either consciously or unconsciously, design the methodology and statistical analysis to produce expected results. Researchers are under constant pressure to publish, in fact many post-doctoral positions are conditional on publishing, and typically only positive results are published. Papers published in top journals facilitate future publication, which means the cycle of irreproducibility is perpetuated.

Within the research rat race of academia, it often seems to be a waste of time and effort to apply for grant funding with a hypothesis that is in direct contradiction of already published articles in leading journals. Researchers are more likely to be remembered for startling discoveries than disproving an existing publication.

The reasons governing which research topics are likely to receive funding are interesting. [3 4]

The winner of the 2013 Nobel Prize for Medicine and Physiology – Randy W Schekman – publicly stated that he will boycott three top journals because "*pressure to publish in 'luxury' journals encourages researchers to cut corners and pursue trendy fields in science instead of doing more important work*." Dr Schekman maintains that research is judged by the impact factor of the journal it is published in and that these journals only accept papers that "*will make waves because they explore sexy subjects or make challenging claims*." The impact factor does not reflect the quality of the research and encourages fashion trends in research rather than promoting replication studies.[4]

Perhaps the answer to this dilemma is web-based, open-access journals with no subscription fee which publish all papers which meet certain quality standards with no caveats about positive or negative results or replication studies. [4]

How has it come about that academic research is perceived as so fundamentally flawed that even publication in an elite journal cannot guarantee the quality of the

research? It could be a series of factors that are committed, either unconsciously or consciously, which compounded together render research irreproducible.
For example:

I. Bias – how many researchers can say honestly that if they don't get the expected results (which fit their hypothesis) they repeat the experiment until the results are as expected?
II. Results – likewise, if the results are as expected how many researchers repeat the experiment to prove it wasn't a fluke? Are results cherry-picked to fit the hypothesis?
III. Controls – how many researchers can confirm that they always use appropriate positive and negative controls?
IV. Reagents – all reagents used must be validated. This is reflected in the fact that analytical grade chemicals from reputable suppliers are used in laboratories. Antibodies, however, are a different matter. The onus is on the researcher to validate the antibody they are using. How many researchers take the time to validate the antibodies they are using?
How many generic emails are circulated round academic mailing lists begging for a vial of a particular cell line when it is next being split or a few microlitres of a particular antibody that will be replaced when their order arrives? How reliable would these be in a research study – there is no provenance - and how are they written into a publication?

To add to the above points, pressure is put on junior researchers to produce "good results". [4] [5] The Principal Investigator (PI) is not interested how these results were obtained, only that they are as expected. This puts enormous pressure on junior researchers to produce expected results by any means possible.
Booth also comments on the quality of the Material and Methods section of scientific publications and the reluctance of researchers to fully disclose how the experiments were done and using exactly which reagents.[5]
He reiterates the point made above that states that only the "best results" are used and contradictory data is ignored.

What is quality?
It is defined in the Oxford Dictionary as "*the standard of something as measured against other things of a similar kind; the degree of excellence of something".*

Waddell further divides "quality" into:

a) Quality control (QC) – this is quantitative and can be applied throughout the study by the researcher and any other study personnel.
b) Quality assurance (QA) – this is qualitative and should be conducted pre, during and post research by independent, appropriately qualified QA personnel. [6]

This thesis addresses the hypothesis that quantitative expression of protein expression can be used to identify putative biomarkers of gemcitabine and 5-FU resistance in pancreatic and colorectal cancers, and that the methodology is transferrable to other disease types.

Aim of the study

The aim of this study is to analyse a range of biomarkers which may be associated with gemcitabine and 5-FU resistance. The results of this study could have major implications for the patient population if it were found that one or several of these biomarkers were clinically relevant to survival time and/or resistance. At best, patients' survival could be prolonged; at worst, patients would not be subjected to the debilitating side-effects of chemotherapeutic drugs.

With this in mind, this project used Quality Management wherever feasible to ensure that the conduct of the study and the results produced were both accurate and reproducible.

1.2 Biology of normal colorectal tissue

COLON

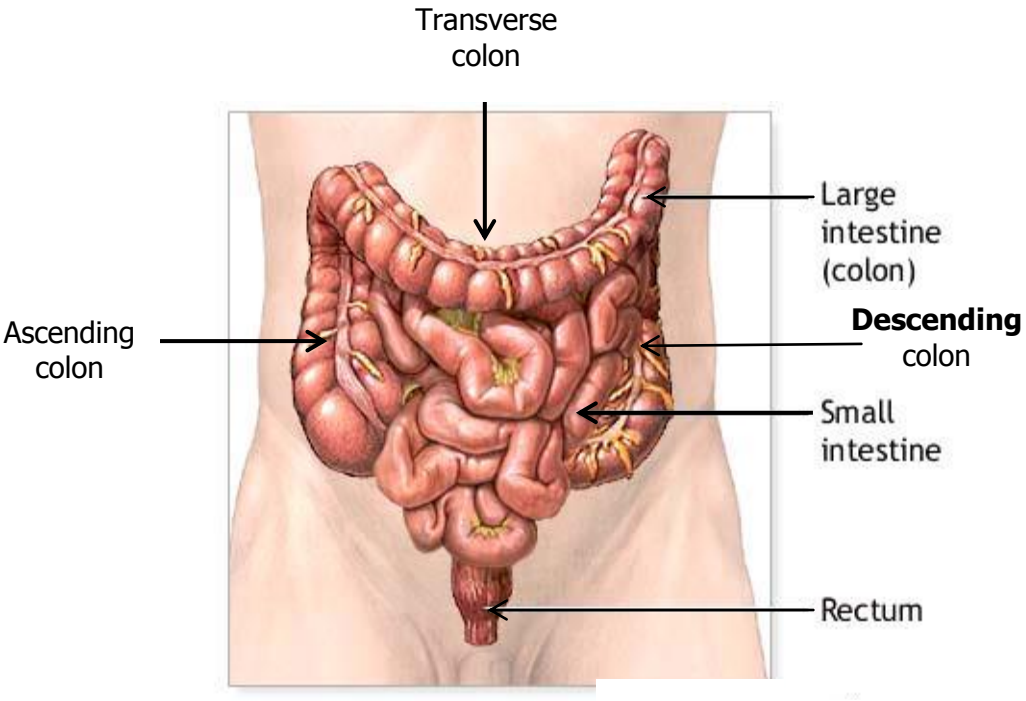


Figure 1.1 The colon and rectum [7]

If the large and small intestines were laid out in a straight line they would measure around 7m in a normal human adult. The large intestine is so named because it is wider in diameter when compared to the small intestine. It measures 1.5m when laid out straight with a diameter of 7.5cm. The small intestine is much longer – around 7m in length – with an average diameter of 2.5cm. [8]

Normal colon consists of four basic layers: mucosa, sub mucosa, muscularis externa and serosa.

The mucosa layer consists of three layers:

1) Muscularis mucosa – consists of two thin layers of smooth muscle.

2) Lamina propria – consists of a thin layer of loose connective tissue. There are no villi but numerous tubular glands which duct on to the surface epithelium and secrete mucus and other serous secretions.

3) Surface epithelium – simple columnar epithelium which contains very few goblet cells.

The sub mucosa layer contains no plicae but does include lymph nodules.

The muscularis externa layer consists of two layers:

i) Outer longitudinal layer consisting of three bands of muscle fibres called taeniae.

ii) Inner circular layer, which is a thin layer of muscle.

The serosa layer is the outermost layer and consists of areolar tissue, which is continuous with the mesentery supporting the gut.

Figure 1.2 Normal colon stained with haematoxylin and eosin [9]

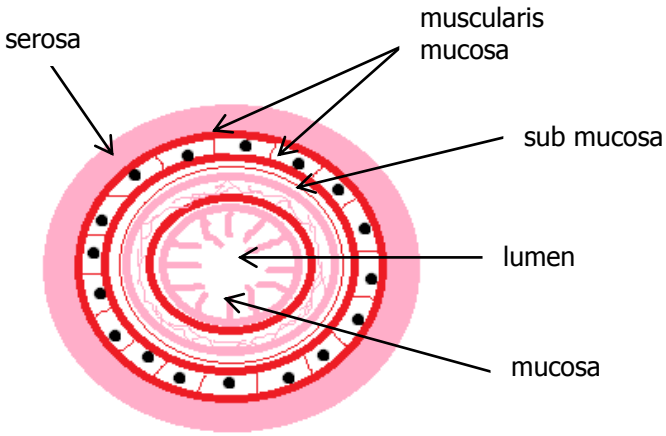


Figure 1.3 Transverse section of colon

RECTUM

The rectum is the final portion of the large intestine. It is approximately 12cm in length and consists of the same four basic layers as colon: mucosa, sub mucosa, muscularis externa and serosa.

The mucosa layer consists of three layers:

1) Muscularis mucosa – consists of two thin layers of well-developed smooth muscle. Lamina propria and sub mucosa merge in the rectum.

2) Lamina propria – consists of a thin layer of loose connective tissue which is much thicker than in the colon.

3) Surface epithelium – this becomes stratified squamous towards the recto-anal junction where it becomes longitudinal folds called columns of Morgagni.

The sub mucosa layer contains few lymph nodules. In the anal canal there is a good supply of small veins which can dilate and bulge into the lumen – haemorrhoids.

The muscularis externa layer consists of two layers.

1) Outer longitudinal layer with no taeniae.

2) Inner circular layer, which is a thin layer of muscle.

The muscularis externa layer in rectum is much thicker than that in colon.

The serosa layer is the outermost layer and consists of areolar tissue which is continuous with the mesentery supporting the gut.

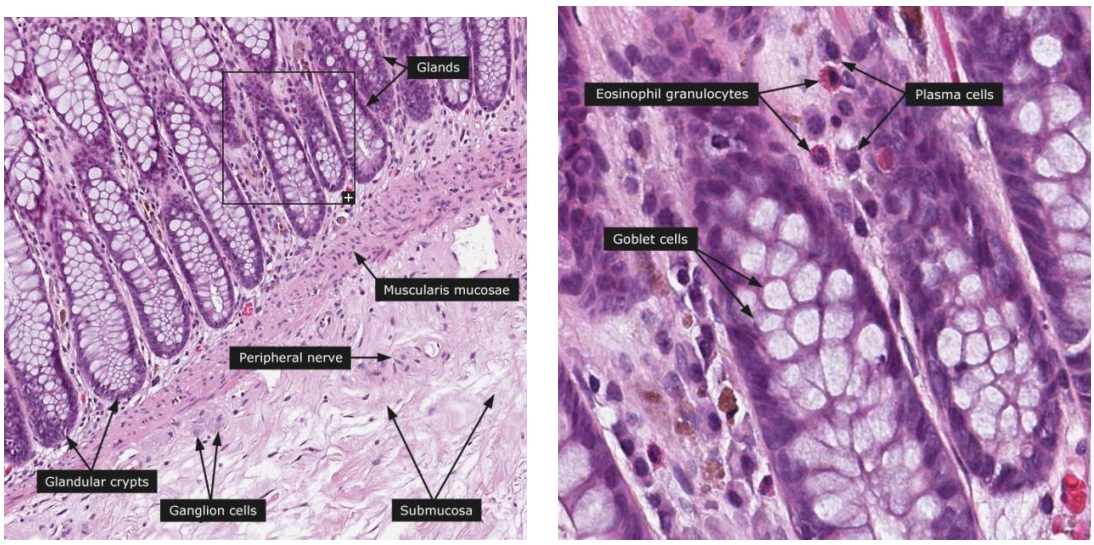


Figure 1.4 Normal rectum stained with haematoxylin and eosin (x10 and x20) [10]

Malignancy

Malignant is defined in the Oxford English Dictionary as *"… (of a neoplasm): having the property of uncontrolled growth, with loss of differentiation, invasion and destruction of local tissue, and (often) metastasis to distant sites. Also: of the nature of or caused by such a neoplasm."*

In order to appreciate and understand malignancy it is necessary to study and be cognizant of the structure and function of normal tissues and organs.

1.2.1 Colorectal cancer

Colorectal cancer is the third most common cancer for both males and females in Scotland (in 2012) and makes up 13% of all cancers. [2]

The biggest challenge faced by oncologists today is predicting which patients will respond to treatment. 5-fluorouracil (5-FU) is the first line treatment for colorectal cancer, used both singly and in combination with other drugs; however, the response rate is around 20% when used singly and just under 50% when used in combination. [3 11]

1.2.1.1 Incidence and survival rates of colorectal cancer

Colorectal cancer is the second most common cause of tumour-related death in the UK. It is the third most common cancer in the world and accounts for 10% of all

cancers worldwide. It is the third most common cancer in both males and females in the UK – 13% of all cancers – accounting for 11% of all cancers. Around 66% of colorectal cancers originate in the colon and around 34% originate in the rectum according to UK statistics. 63% of rectal cancers occur in males whereas colon cancer has an even male/female distribution.[12]

Colorectal Cancer		England	Wales	Scotland	Northern Ireland	UK
Male	**Cases**	**18,590**	**1,421**	**2,177**	**646**	**22,834**
Female	**Cases**	**14,628**	**933**	**1,790**	**510**	**17,861**
Persons	**Cases**	**33,218**	**2,354**	**3,967**	**1,156**	**40,695**
Crude rate = incidence per 100,000 people						
Male	**Crude Rate**	**72.2**	**96.6**	**86.0**	**73.0**	**74.5**
Female	**Crude Rate**	**55.2**	**60.8**	**66.5**	**55.7**	**56.5**
Persons	**Crude Rate**	**63.6**	**78.3**	**76.0**	**64.2**	**65.4**

Table 1.1 New cases and rates of colorectal cancer in the UK in 2010 [13]

Duke's A colorectal cancer has a 5-year relative survival rate of over 90% whereas for Duke's D colorectal cancer, the 5-year relative survival rate is under 7%. Surgical excision is the optimal treatment for non-metastatic patients whilst surgical resection and adjuvant chemotherapy is recommended for advanced stage patients. [14]

Duke's stage at diagnosis	Percentage of cases	5-year relative survival
A	**8.7%**	**93.2%**
B	**24.2%**	**77.0%**
C	**23.6%**	**47.7%**
D	**9.2%**	**6.6%**
Unknown	**34.3%**	**35.4%**

Table 1.2 Percentage of cases and 5 year relative survival (%) by Duke's stage at diagnosis, colorectal cancer patients diagnosed 1996-2002, England [14]

The Scottish Bowel Screening Programme was implemented by all Scottish health boards by December 2009. Up to 31st October 2010 in the Scottish Bowel Screening Programme:

- Just fewer than 1,500,000 individuals were invited to take part
- Over 800,000 took up the offer and achieved a final result
- Over 1400 cancers were diagnosed through screening

Therefore, approximately half of individuals invited to take part in the screening programme responded and of this half, approximately 3% were diagnosed with colorectal cancer.[15]

1.2.1.2 Location and histological sub-types of colorectal cancers

Colorectal cancer may be divided into categories based on the location and histological characteristics of the tumour. Colon cancer can initially be divided into right sided and left sided. Research has been conducted on right versus left sided colon cancer with the conclusion that right-sided colon cancers have less overall survival times than left-sided cancers. [16] [17] Colon cancer can also be categorised into actual location of the tumour: ascending colon, descending colon, caecum, distal sigmoid colon, distal transverse colon, hepatic flexure, ileocaecal valve, left colonic phlegmon, mesorectum, mid transverse colon, proximal transverse colon, rectosigmoid, rectum, sigmoid colon, splenic flexure and transverse colon.
The WHO classifies colorectal tumours as follows:

Epithelial tumours		Non-epithelial tumours
Adenocarcinoma	**Tubular**	**Lipoma**
	Villous	**Leiomyoma**
	Tubulovillous	**Gastrointestinal stromal tumours**
	Serrated	**Leiomyosarcoma**
Carcinoma	**Adenocarcinoma**	**Angiosarcoma**
	Mucinous adenocarcinoma	**Kaposi sarcoma**
	Signet-ring cell carcinoma	**Malignant melanoma**
	Small cell carcinoma	**Malignant lymphoma**
	Squamous cell carcinoma	
	Adenosquamous carcinoma	
	Medullary cell carcinoma	
	Undifferentiated carcinoma	

Table 1.3 WHO classification of tumours of the colon and rectum [18]

Adenocarcinoma is the most common type of colorectal cancer which accounts for 90-95% of all colorectal cancers.
Adenocarcinomas can be either mucinous or non-mucinous. The mucinous subtype accounts for 10-15% of all adenocarcinomas.

1.2.1.3 Risk factors in colorectal cancer

(i) **Age and sex** – the risk of developing colorectal cancer increases directly with age. The amount of cases for males peaks between the ages of 70 and 79, before declining sharply in the early to late eighties.
For females, there is a gradual increase up to age 75 after which the number of cases remains more or less static. [19]

(ii) **Diet** – there is increasing evidence that eating a diet rich in red and processed meat increases the risk of developing colorectal cancer [20] [21]; however there is no convincing evidence that fish consumption, in particular fresh or salted fish, reduces the risk. [22]

A high fibre diet, especially from cereals and whole grains, has been shown to protect against colorectal cancer. No association was found with fibre from fruit and vegetables. [23] In fact, the risk reduction from a diet high in fruit and vegetables is negligible, although eating garlic – both cooked and uncooked – may be associated with a reduction in risk. Evidence is unclear as garlic is very rarely eaten as a single food item, therefore it could be the reduction in risk only occurs when consumed with other vegetables or herbs. There has been no benefit seen from garlic supplements. [24]

There is no conclusive association between a high fat diet and colorectal cancer. [21]
An increased daily intake of milk reduces the risk of colorectal cancer. It is thought that the effect from milk is likely to be related to calcium. [25]
Any evidence that high sugar consumption is a risk factor is very limited. [26]

(iii) **Obesity** – the risk of colorectal cancer increases with BMI, or more specifically waist size. The risk is greater in men than women. [27] [28]

(iv) **Exercise** – the risk of colon cancer reduces as one exercises but the risk for rectal cancer does not change. [29] [30]

(v) **Alcohol and smoking** – moderate alcohol consumption increases the risk of colorectal cancer, the risk increasing directly proportionally to alcohol intake.
A moderate amount of alcohol is anything more than a small glass of wine or half a pint of beer per day. [31]

The risk of colorectal cancer increases with the number of cigarettes smoked per day. Ex-smokers are at greater risk than people who have never smoked. Smoking increases the risk of developing rectal cancer more than colon cancer. [32]

(vi) **Nonsteroidal anti-inflammatory drugs** – low dose aspirin (75mg/day) has been shown to significantly reduce the risk of colorectal cancer. [33]

(vii) **Statins** – the evidence on statin use remains unclear however, a recent study showed that using statins for 4 years or more increased the risk of colorectal cancer. [34]

(viii) **Hormone replacement therapy (HRT)/oral contraceptives (OC)** – the evidence is mixed for HRT however risk is reduced for women who have ever taken OCs, the risk reduction being greater the more recent the use. [35]

(ix) **Inflammatory bowel disease** – the risk of developing colorectal cancer is increased in people with ulcerative colitis and Crohn's disease, the risk increasing with the years of suffering the disease. [36]

(x) **Diabetes** – research has shown an increased risk in people with type II diabetes patients. However, metformin is associated with a decrease in risk. [37] [38]

(xi) **Human papilloma virus (HPV)** – approximately 90% of invasive anal cancers are linked to HPV infection, with HPV16 being the most common type. [39]

(xii) **Radiation** – more than 1% of all colorectal cancers in the UK can be linked to radiation exposure. [40]

(xiii) **Family history** – hereditary factors account for around 20% of colorectal cancers with a direct 5% attributed to familial adenomatous polyposis (FAP), polyposis syndromes and hereditary non-polyposis colorectal cancer (HNPCC). Although patients with FAP have almost 100% risk of developing colorectal cancer by the age of 40, this population accounts for less than 1% of all colorectal cancers. [41]
Patients with HNPCC account for 1-4% of all colorectal cancers.
Other hereditary factors account for another 5% of colorectal cancers. [42]

1.2.1.4 Diagnosis of colorectal cancer

The diagnosis of any cancer is predominantly as a result of a patient presenting to their GP with a symptom or symptoms that are not a feature of their everyday life. As previously stated, the Scottish Bowel Cancer Screening Programme identifies that 3% of all persons screened will be diagnosed with colorectal cancer. For those who are too young or decline to take part in the screening programme, there will be symptoms of the disease that lead to a diagnosis being made.

The symptoms of colorectal cancer are: [43]

- Rectal bleeding
- Abdominal pain
- Altered bowel habits
- Anaemia
- Weight loss
- Anorexia
- Fatigue
- Nausea or vomiting
- Tenesmus
- Mucous in stools
- Rectal pain
- Obstruction

If colorectal cancer is suspected then a variety of methods are utilised to investigate:

1) Digital rectal examination – this is usually performed by the GP to ascertain whether there is a palpable lump within the rectum.

2) Colonoscopy – a colonoscope is inserted into the rectum and manoeuvred throughout the length of the colon. A small camera and light are attached to the end of the colonoscope which means images can be taken. A small biopsy sample may also be taken during this procedure.

3) Sigmoidoscopy – this is identical to a colonoscopy except that the tube inserted is much shorter and can only be used to examine the rectum and part of the colon.

4) Computerised Tomography/Magnetic Resonance Imaging (CT/MRI) scans – these scans are used to give a detailed depiction of the colorectal area. They may also be used to determine if there are any metastases in other organs.

1.2.1.5 Staging of colorectal cancers

Colorectal cancer is staged using the TNM system and also the Duke's classification.

As with all cancers, staging is used to describe the extent and severity of disease and may facilitate treatment plans and indicate the prognosis of the patient.
The TNM system looks at the size of the primary tumour (T), whether there is evidence of cancer cells in the lymph nodes (N) and whether the cancer has spread to other parts of the body (M). Duke's staging is used only for colorectal cancer and compares with the TNM system as shown in the table below.

Stage	T	N	M	Duke's classification
I	T1	N0	M0	A
	T2	N0	M0	B1
II	T3	N0	M0	B2
	T4	N0	M0	B2
III	T1, T2	N1, N2	M0	C1
	T3, T4	N1, N2	M0	C2
IV	Any	Any	M1	D

Table 1.4 Comparison of TNM staging with Duke's classification [44] [45]

See Appendix 1 for staging and grading of colorectal tumours.

1.2.1.6 Treatment of colorectal cancer

Once a diagnosis has been confirmed, treatment options are determined by the stage, grade and location of the tumour, the patient's age and general health. Other factors that will influence treatment are the biological and genetic properties of the cancer cells.
The options available are surgery, radiotherapy and chemotherapy; often a combination of all three.

Surgery

In early stage cancers surgical resection is a method used to potentially remove the entire tumour. This can be ameliorated by pre-surgery radiotherapy to shrink the tumour and post-operative chemotherapy to treat any potential cells left *in situ*.
The most important factor in this type of surgery is the distance between the resection margin and the tumour and the presence/absence of any residual tumour.

Radiotherapy

Often used for rectal tumours both pre- and post-surgery and given in combination with chemotherapy to either reduce tumour size pre-surgery or treat any remaining cancer cells post-surgery.

Chemotherapy

The standard chemotherapy first-line treatment for colorectal cancer is 5-fluorouracil (5-FU). This is despite the fact that the response rate is less than 20% when used as a monotherapy. The response rate rises to 20-30% when leucovorin (folinic acid) is administered at the same time. When oxaliplatin is added to the treatment the response rate increases to just less than 50%. 5-FU may also be given in combination with irinotecan. [3]

Capecitabine is a prodrug which may be given orally, and is converted to 5-FU within the tumour cells. 5-FU is delivered intravenously.

Adjuvant chemotherapy may be offered to patients depending on the stage of the disease and the success of the resection. The most common drugs used are:

- FOLFOX – a combination of folinic acid, 5-fluorouracil and oxaliplatin given intravenously
- Capecitabine – given orally
- Fluorouracil (5-FU) as a monotherapy – given intravenously
- XELOX or CAPOX – a combination of oxaliplatin and capecitabine [46]

1.2.1.7 Metastasis of colorectal cancer

Fewer than 25% of people who develop colorectal cancer will ultimately have metastasis to the liver. As the liver metastases rather than the primary tumour determine the prognosis of the patient, it is important to check the concurrence of protein expression in both tumour sites. [47] Research has shown that although the primary tumour may respond to chemotherapy, the metastatic tumour is more resistant. [48] The proteins associated with the metabolism of 5-FU are thymidine phosphorylase, dihydropyrimidine dehydrogenase, ribonucleotide reductase subunit M1, ribonucleotide reductase subunit M2 and thymidilate synth(et)ase.

1.3 Biology of normal pancreatic tissue

PANCREAS

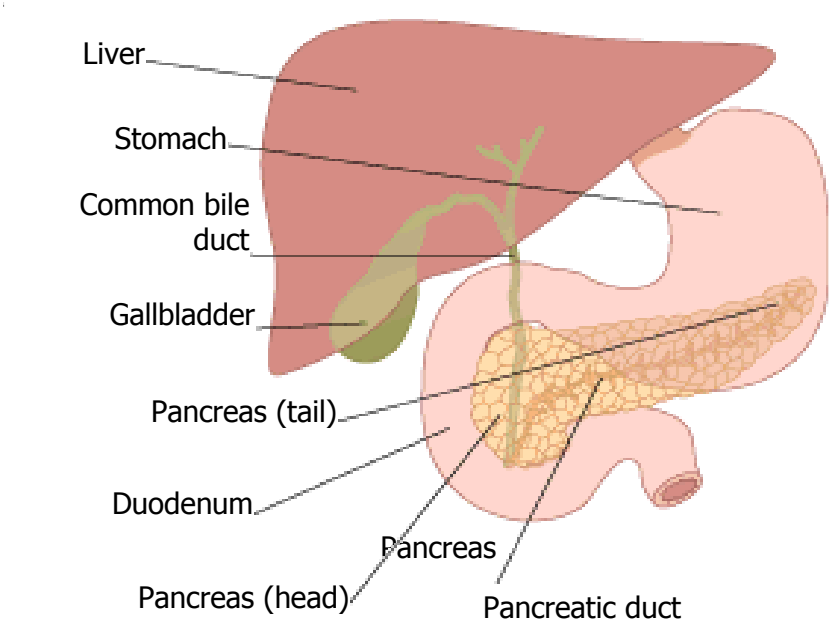


Figure 1.5 The site of the pancreas within the body showing the proximity to other organs [49]

The pancreas is located just behind the stomach. The head of the pancreas nestles in the curve of the duodenum, the body of the pancreas lies in close proximity to the liver and the tail of the pancreas is closest to the spleen. The uncinate process is the part of the pancreas that bends backwards and underneath itself.

The pancreas is approximately 6 inches long and shaped like a flattened pear.

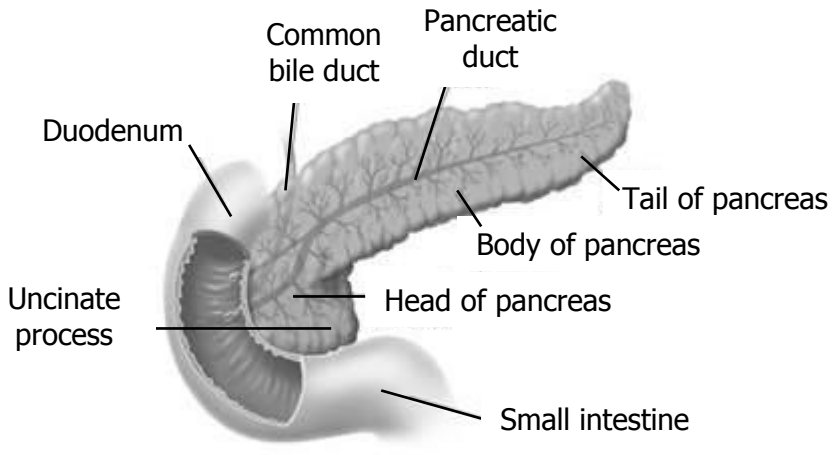


Figure 1.6 Gross structure of the pancreas [50]

The pancreatic duct runs the length of the pancreas with many smaller ducts branching off. The pancreatic duct merges with the common bile duct at the entrance to the duodenum to form the ampulla of Vater.

The pancreas has two distinct components with their own unique function, the endocrine and exocrine parts. The endocrine substances produced are secreted directly into the bloodstream.
The exocrine part of the pancreas contains glands which produce digestive enzymes. It contains two major types of cells:
Acinar cells – these produce digestive enzymes in an inactive form called zymogens, this is to prevent the pancreas digesting itself. The most common enzymes include pancreatic protease, amylase and lipase.
Duct cells – these produce bicarbonate which mixes with the zymogens to form pancreatic juice which flows into the duodenum. The zymogen is only activated once it reaches the intestine and the bicarbonate neutralises acid entering the small intestine from the stomach.

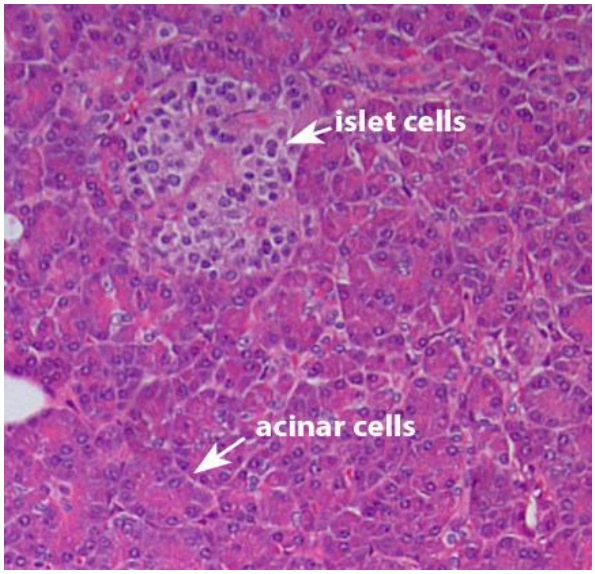


Figure 1.7 Normal pancreas stained by haematoxylin and eosin [51]

Malignancy

Malignant is defined by the National Cancer Institute as "*A term for diseases in which abnormal cells divide without control and can invade nearby tissues. Malignant cells can also spread to other parts of the body through the blood and lymph systems. Also called cancer*".
In order to appreciate and understand malignancy we must first study the structure and function of normal tissues and organs.

1.3.1 Pancreatic cancer

Pancreatic cancer is the tenth most common cancer in the UK and makes up 2.55% of all cancers. [52]

The biggest challenge faced by oncologists treating patients with pancreatic cancer is the limited clinical action of gemcitabine on the tumour. Gemcitabine is the first line treatment for pancreatic cancer, used both singly and in combination with erlotinib. Only around 10% of pancreatic tumours are suitable for potential resection, which combined with adjuvant chemotherapy results in a 5-year survival rate of less than 20%. [53]

1.3.1.1 Incidence and survival rates of pancreatic cancer

Pancreatic cancer is the fifth most common cause of tumour-related death in the UK. It accounts for 2% of all cancers worldwide. While it is the twelfth most common cancer in males in the UK – less than 3% of all cancers – it is the eighth most common cancer in females in the UK, accounting for 3% of all cancers. Around 95% of pancreatic cancers originate in the exocrine part of the pancreas and the majority of these are ductal adenocarcinomas. Tumours arising from the endocrine part of the pancreas are rare. [54]

Pancreatic Cancer		England	Wales	Scotland	Northern Ireland	UK
Male	Cases	3,497	260	343	89	4,189
Female	Cases	3,561	260	353	100	4,274
Persons	Cases	7,058	520	696	189	8,463
Crude rate = incidence per 100,000 people						
Male	Crude Rate	13.6	17.7	13.6	10.1	13.7
Female	Crude Rate	13.4	16.9	13.1	10.9	13.5
Persons	Crude Rate	13.5	17.3	13.3	10.5	13.6

Table 1.5 New cases and rates of pancreatic cancer in the UK in 2010 [55]

The average survival time for a pancreatic cancer patient following surgical resection is approximately 11-20 months. This drops to 6-11 months in unresectable localised

tumour patients. For patients with unresectable, metastatic disease the average survival time is 2-6 months. [56]

Less than 20% of patients have resectable tumours at initial diagnosis of the disease.

Survival time	Relative survival % Males	Relative survival % Females	Relative survival % Total
1 year	15.3	16.1	15.7
3 years	4.8	4.5	4.6
5 years	3.6	2.9	3.2
10 years	-	-	-

Table 1.6 Relative survival rates for pancreatic cancer for patients diagnosed 1983-2007 [57]

The above table shows that in Scotland between 1983 and 2007, only around 15% of pancreatic cancer patients were still alive after one year and around 3% were still alive after 5 years. No patients were alive at the 10 year milestone. These statistics are shocking and due mainly to the fact that pancreatic cancer is diagnosed at a late stage of the disease. The first symptoms with which most patients present to their GP are jaundice, both with and without abdominal pain.

There is no screening programme in place for pancreatic cancer, possibly due to costs (a CT scan would be the most effective method of screening) and the relatively small portion of the population who are affected by pancreatic cancer.

EUROPAC is the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer. [58] They state that 5-10% of pancreatic cancers are hereditary and that, although many genes could be involved, the only one identified thus far is the BRCA2 gene. This organisation offers a screening service for people over 40 who are deemed high risk, but each patient is considered on a case-by-case basis. The following methods may be used to screen patients:

Fasting glucose – this is done to check the endocrine part of the pancreas is functioning normally.

CA19-9 – this is a cancer antigen that is tested via a blood sample. The drawback to this test is that it is not specific to pancreatic cancer, merely an indicator when evaluated alongside additional tests.

Endoluminal ultrasound (EUS) – a small camera is inserted into the stomach via the gullet and images of the pancreas obtained.

CT scan – a scan that produces a 3-D image of the pancreas.

Endoscopic Retrograde Cholangio-Pancreatography (ERCP) – very similar to EUS but a sample of pancreatic juice is taken for molecular analysis. [59]

1.3.1.2 Location and histological sub-types of pancreatic cancer

Pancreatic cancer may be divided into categories based on the location and histological characteristics of the tumour. Around 65% of cancers arise in the head and 30% arise in the body or tail of the pancreas. The remaining 5% engulf the whole of the pancreas.

Pancreatic ductal adenocarcinomas account for 85-90% of all pancreatic cancers and endocrine tumours account for less than 5% of all pancreatic cancers. Acinar cell carcinomas make up 1-2% of all pancreatic cancers and the remaining types are relatively rare. [60]

1.3.1.3 Risk factors in pancreatic cancer

(i) **Age and sex** – The number of cases reported in Scotland peaks for males between the ages of 70 and 74 before gently declining thereafter.

For Scottish females reported cases peak between the ages of 80 and 84 and remain high. [61]

(ii) **Ethnicity** – Cases of pancreatic cancer are higher in persons of African descent or Ashkenazi Jews. The reasons for this could be the high rates of smoking, diabetes and obesity in Africans and the prevalence of BRCA2 gene mutation in Ashkenazi Jews. [62]

(iii) **Smoking and alcohol** – smokers are 2 to 3 times more likely to develop pancreatic cancer than non-smokers. Heavy drinkers – defined as 3 or more units of hard liquor per day – are 36% more likely to develop pancreatic cancer. [63] [64]

(iv) **Diet and obesity** – the risk of developing pancreatic cancer increases with increased BMI. Clinical research has shown that red meat consumption can increase the risk of pancreatic cancer; however there is no substantial evidence that a healthy diet decreases the risk. [65] [66]

(v) **Chronic pancreatitis** – the risk increases in chronic pancreatitis sufferers by threefold. The risk for inherited pancreatitis sufferers is far stronger, approximately 45 to 70% greater. [67] [68]

(vi) **Stomach ulcer** – the risk is doubled in persons who have a stomach ulcer. This could be related to the production of nitrosamines by bacteria present in ulcerated stomachs. [69]

(vii) **Inflammatory bowel disease** – long term inflammatory bowel conditions, e.g. ulcerative colitis or Crohn's disease, increases the risk of pancreatic cancer, in some cases by 75%. [70] [71]

(viii) **Gum disease** – recent research has shown a link between gum disease and increased risk of pancreatic cancer, however it is unclear why. [72]

(ix) **Skin allergies** – some research has shown that certain skin allergies such as eczema and hives can reduce the risk of pancreatic cancer. This could be due to the fact that people who suffer from allergies have a more active immune system than those who don't. [73]

(x) **Family history** – familial syndromes and genetic conditions account for 10% of all pancreatic cancers.

- Hereditary pancreatitis is caused by germline mutations on the cationic trypsinogen 7q35 gene on and causes recurring bouts of acute pancreatitis. Pancreatitis is a risk factor for pancreatic cancer, therefore people with this condition have an increased risk of pancreatic cancer. [74]
- Familial atypical multiple mole melanoma (FAMMM) syndrome is caused by germline mutations on the p16 gene on 9p and increases the risk of developing both melanoma and pancreatic cancer. [75]
- Peutz-Jeghers syndrome is an autosomal dominant disorder caused by mutations on the serine/threonine kinase gene STK11(LKB1). Sufferers of this syndrome have a 35% increased risk of developing pancreatic cancer. [76]

1.3.1.4 Diagnosis of pancreatic cancer

The diagnosis of pancreatic cancer is virtually impossible in the early stages of the disease due to the fact it is symptomless. Most cases are referred by the GP where patients have symptoms which could indicate many different diseases. One of the most common presentations is jaundice and unexplained weight loss. The GP may then check for the following symptoms:

- Jaundice
- Swollen or tender abdomen
- Presence of bile in the urine
- Light coloured stools/darker than usual urine
- Unintentional weight loss
- Eating/digesting food problems
- Loss of appetite
- Vomiting
- Fatigue
- Diabetes
- Itchy skin
- Fever

See Appendix 2 for diagnosis, staging and grading of pancreatic tumours.

1.3.1.5 Treatment of pancreatic cancer

Once a diagnosis has been confirmed, treatment options are determined by the stage, grade and location of the tumour, the patient's age and general health. Other factors that will influence treatment are the biological and genetic properties of the cancer cells. The options available are surgery, radiotherapy and chemotherapy; often a combination of all three.

Surgery

Surgery for pancreatic cancer can be either curative or palliative. The decision as to which type of surgery, if any, is determined by the size and location of the tumour and also the stage of the cancer. Fitness to undergo surgery is also taken into consideration. Surgical resection is only possible in 15-20% of cancers. The most common pancreas surgery is a Whipple's procedure and this is used for tumours in the head of the pancreas

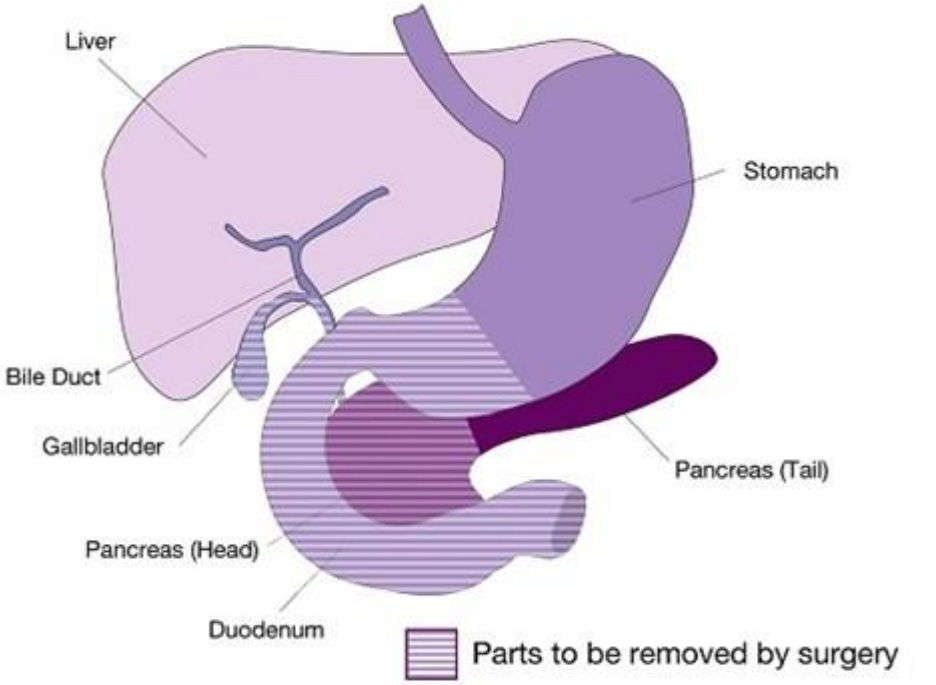


Figure 1.8 Pancreas pre-Whipple's procedure [77]

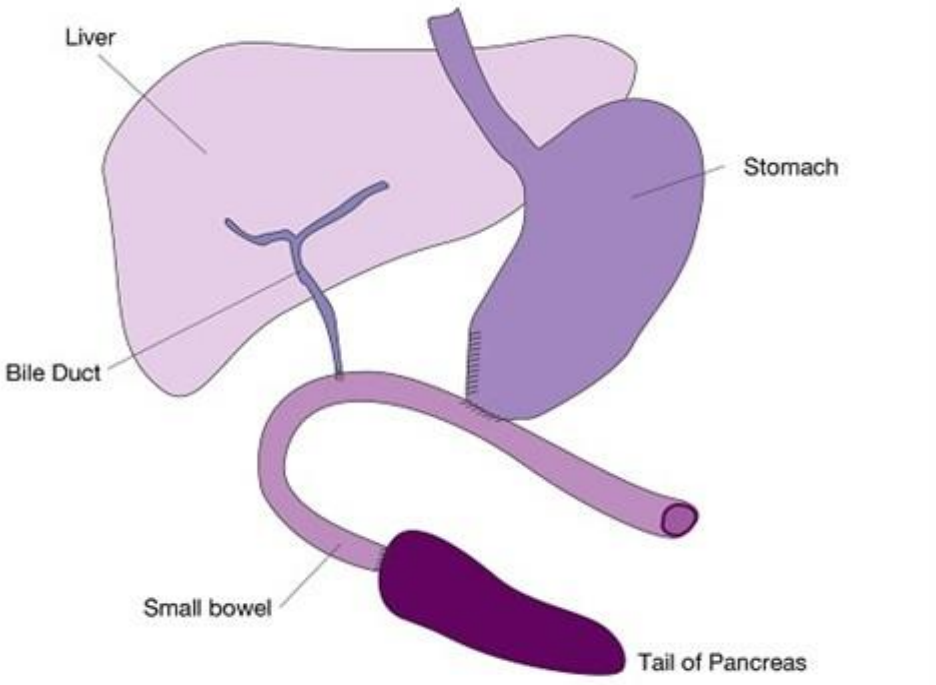


Figure 1.9 Pancreas post-Whipple's procedure [77]

For tumours occurring in the tail of the pancreas a distal pancreatectomy may be performed. This involves removal of the body and tail of the pancreas, and often the spleen is removed too.

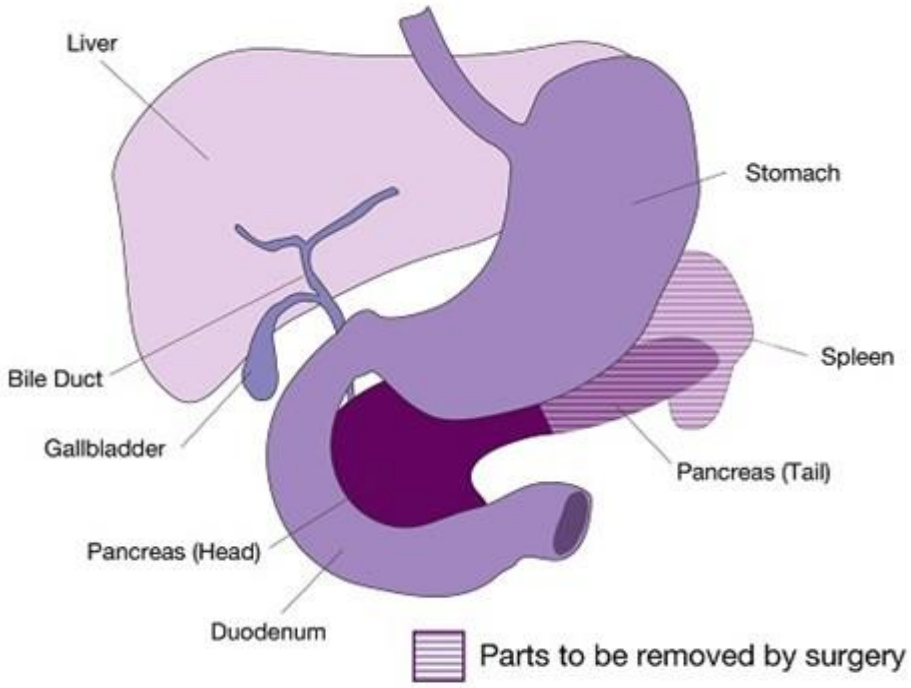


Figure 1.10 Pancreas pre-distal pancreatectomy [77]

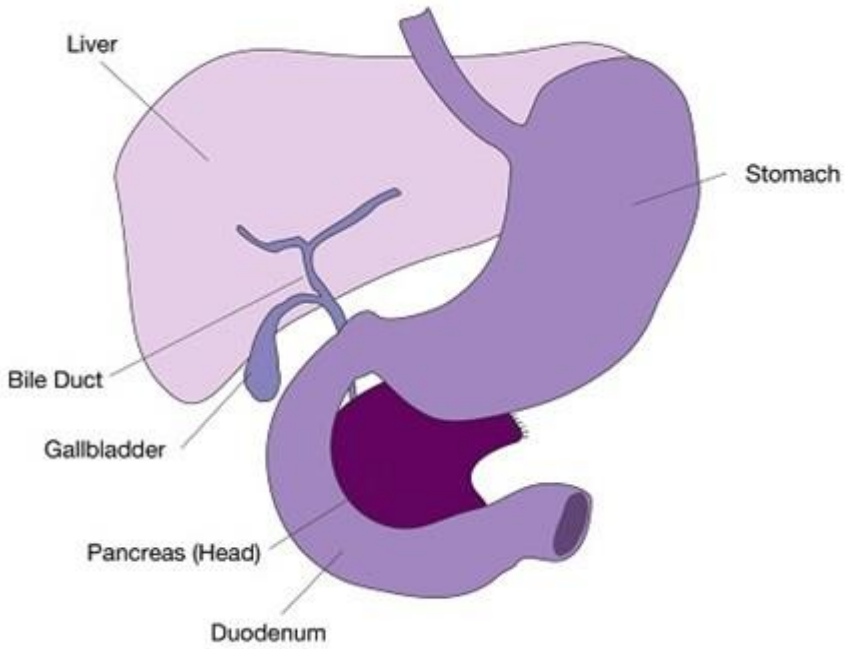


Figure 1.11 Pancreas post-distal pancreatectomy [77]

If resection is not feasible then a stent may be fitted to allow the flow of bile or stomach contents.

Radiotherapy

Radiotherapy may be used both singly and in combination with chemotherapy. It can also be used palliatively to allay pain by shrinking tumours that may be pressing on nerves or blood vessels or causing a blockage.

Chemotherapy

The standard chemotherapy first-line treatment for pancreatic cancer is gemcitabine or 5-fluorouracil (5-FU). Capecitabine may also be given instead of 5-FU. [78]

The ESPAC I and III (European Study group for Pancreatic Cancer) clinical trials compared adjuvant chemotherapy with chemoradiotherapy. The outcome of these trials resulted in a change in standard clinical practice favouring adjuvant chemotherapy. [79] The ESPAC IV trial is ongoing comparing gemcitabine as a monotherapy with gemcitabine plus capecitabine.

Chemotherapy may be used to shrink the tumour pre surgery.

If the cancer is inoperable then chemotherapy may be given in a bid to shrink the tumour and prolong life. Gemcitabine is the main drug used in these cases, both on its own and in combination with other drugs.

These are the most common drugs used:

- GEMCAP – gemcitabine and capecitabine used in combination have increased response rate and overall survival. This is the first-line treatment for advanced, metastatic cancer.
- FOLFIRINOX – a combination of leucovorin, 5-fluorouracil, irinotecan and oxaliplatin may increase overall survival time, although only by a few months. The side effects of this therapy are more severe and can only be given to people who can withstand them.
- Gemcitabine plus nab-paclitaxel – in September 2013 the FDA approved this drug combination for metastatic pancreatic carcinoma. Nab-paclitaxel is paclitaxel bound to albumin, which facilitates entry of the drug into the cell. Recent research suggests that when used in combination with gemcitabine, nab-paclitaxel may increase the accumulation of gemcitabine within the tumour cells.

When chemotherapy fails often other drugs are used in a bid to slow down tumour growth. There is no second line therapy for pancreatic cancer as such, although recently it has been found that tumours may respond to FOLFOX therapy (folinic acid, 5-fluorouracil and oxaliplatin).

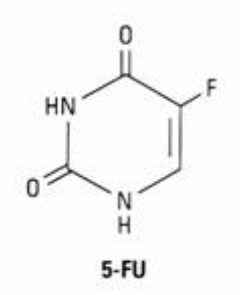
1.4 Drug therapies in colorectal and pancreatic cancer

The 2 drugs which will be investigated in this project are 5-fluorouracil and gemcitabine.

1.4.1 5-fluorouracil (5-FU)

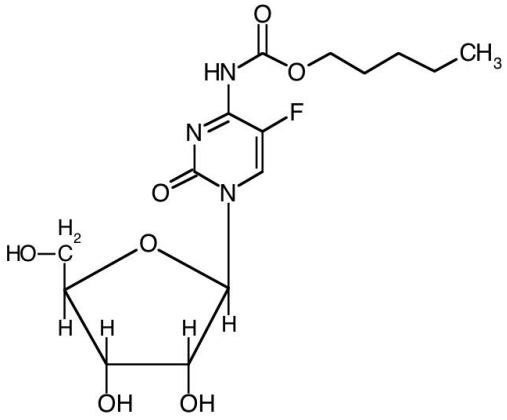
5-fluorouracil, or 5-FU, has been the first line drug for several solid cancers for the past 40 years and remains the first line choice for colorectal cancer; however, the response to 5-FU when used as a monotherapy can be less than 20%. [3] This increases to 20-30% when given in combination with leucovorin and up to 50% when used with irinotecan or oxaliplatin. [11]

1.4.1.1 Structure and metabolism of 5-fluorouracil (5-FU)



5-FU is a pyrimidine analogue which has a similar structure to the pyrimidine molecules of DNA and RNA. Its structure is similar to that of uracil, but there is a fluorine atom at the C5 position instead of hydrogen. As a result of the structure of 5-FU it can be incorporated into DNA and RNA thus leading to cell death.

Figure 1.12 Structure of 5-FU



Capecitabine is a prodrug of 5-FU and can be given orally as opposed to intravenously as is the case with 5-FU.

Capecitabine or 5'-deoxy-5-fluorouridine (5'-DFUR)

Figure 1.13 Structure of capecitabine

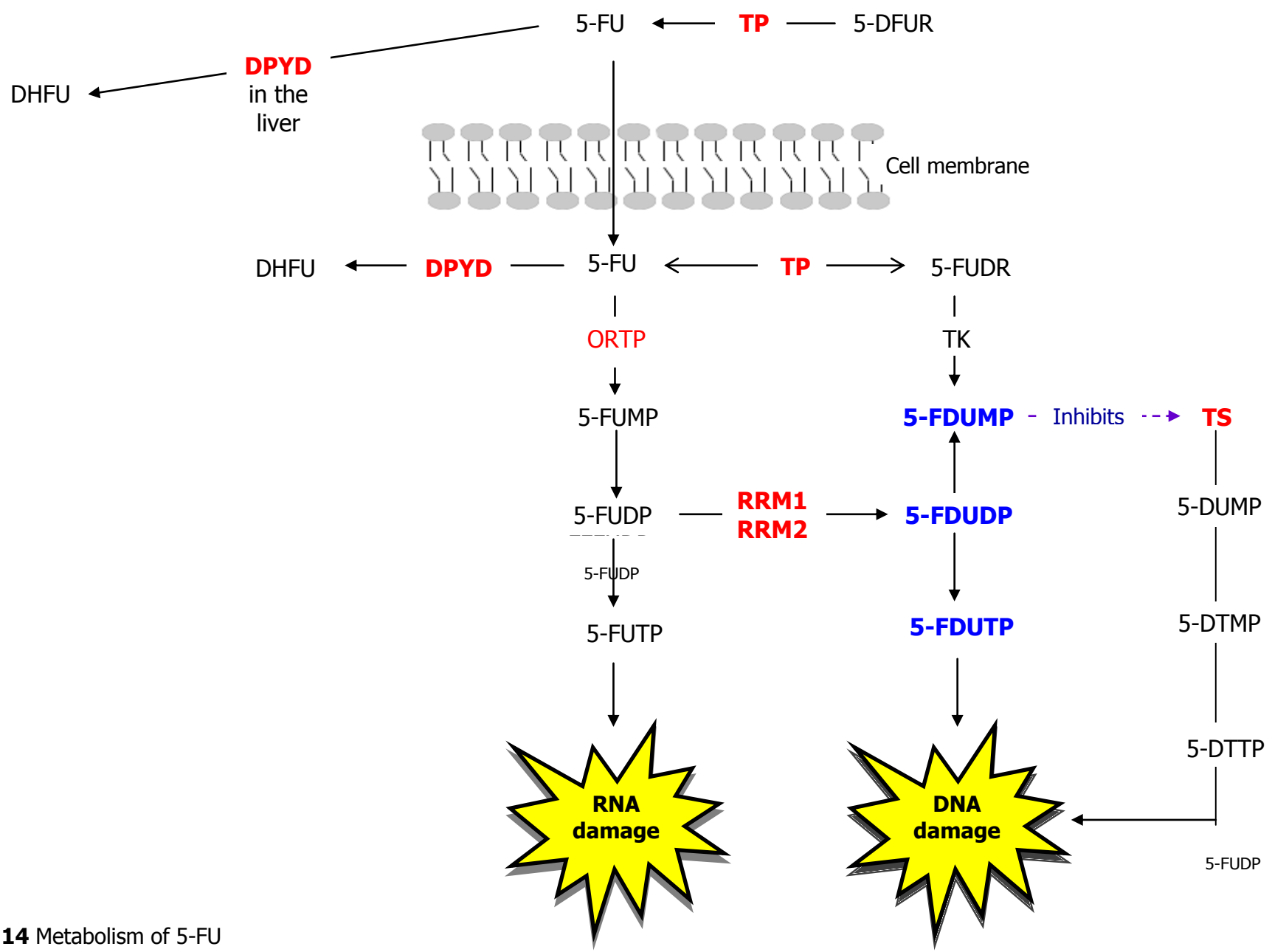


Figure 1.14 Metabolism of 5-FU

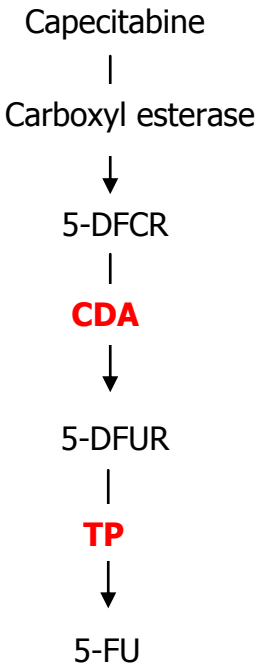


Figure 1.15 Conversion of capecitabine to 5-FU

Key:

5-DFUR – 5-deoxyfluorouridine or doxyfluridine

TP – thymidine phosphorylase

DPYD – dihydropyrimidine dehydrogenase

5-FUDR – 5-fluorodeoxyuridine

DHFU – dihydrofluorouracil

5-FUMP – 5-fluorouracil monophosphate

5-FDUMP – 5-fluorodeoxyuracil monophosphate

ORTP – orotate phosphoribosyltransferase

TS – thymidilate synth(et)ase

TK – thymidine kinase

5-FUDP – 5-fluorouracil diphosphate

5-FDUDP – 5-fluorodeoxyuracil diphosphate

RRM1 – ribonucleotide reductase subunit M1

RRM2 – ribonucleotide reductase subunit M2

5-FUTP – 5-fluorouracil triphosphate

5-FDUTP – 5-fluorodeoxyuracil triphosphate

5-DFCR – 5-deoxyfluorocytidine

CDA – cytidine deaminase

5-DUMP – 5-deoxyuracil monophosphate

5-DTMP – 5-deoxythymidine monophosphate

5-DTTP – 5-deoxythymidine triphosphate

Figure 1.14 shows the conversion of 5-FU to its 3 active states: 5-FDUMP, 5-FDUDP and 5-FDUTP. Figure 1.15 shows the conversion of capecitabine to 5-FU.

About 80% of 5-FU is metabolised to DHFU – an inactive form of drug – in the liver by DPYD.[80] Within both tumour and normal cells, DPYD is the rate-limiting step of 5-FU metabolism. Thymidine phosphorylase (TP) catabolises the conversion of 5-FU to the active substrate 5-FUDR and also catabolises the prodrug 5-DFUR to 5-FU. 5-DUMP works by inhibiting TS, which makes TS an ideal target for chemotherapeutic drugs as it is subsequently phosphorylated to thymidine triphosphate for use in DNA synthesis and repair. Ribonucleotide reductase is an enzyme that produces deoxyoligonucleotides from ribonucleotides, a step that is essential for DNA synthesis and repair. Human ribonucleotide reductase exists in two subunits RRM1 (regulatory) and RRM2 (catalytic).[81 82] Both subunits are necessary for enzymatic activity and are encoded by different genes on separate chromosomes. RRM1 and RRM2 are both critical for DNA synthesis and have been evaluated by several studies as therapeutic targets in various cancers. Importantly, ribonucleotide reductase reduces 5-FUDP to 5-FDUDP. 5-FDUDP is subsequently dephosphorylated into 5-FDUMP, the active 5-FU metabolite which inhibits TS. This pathway operates independent of TP.

1.4.1.2 5-fluorouracil resistance in cancer

5-FU has been the drug of choice to treat colorectal cancer for the past 50 years. This is despite the fact that the response rate is only around 15% when it is used as a monotherapy and peaking at around 40-50% when used in combination with leucovorin. Much research has taken place into the mechanisms of resistance to 5-FU, but there are no definitive markers of resistance which could be used to predict response to the drug. [11]

Dihydropyrimidine dehydrogenase (DPYD) is the rate-limiting step of metabolism of 5-FU into its active state in both tumour and normal cells. Only around 20% of administered 5-FU is metabolised as 80% is broken down by DPYD. It is responsible for converting 5-FU to dihydrofluorouracil (DHFU), an inactive form of the drug. Previous research has shown that intratumoural gene expression levels of DPYD is predictive of response to 5-FU – low DPYD expression equates to increased response. [3 83] Increased levels of DPYD have been shown to be indicative of 5-FU resistance in

both clinical and pre-clinical studies. Upregulated levels of DPYD in the liver have also been correlated with 5-FU resistance.[80] [84]

Positive factors for 5-FU response have been suggested to be gender, favouring females and tumour location, favouring colonic tumours over rectal. [85]

Thymidine phosphorylase (TP) is involved in the conversion of 5-FU to 5-fluorodeoxyuridine (5-FUDR) and also 5-deoxyfluorouridine (5-DFUR) to 5-FU. Research is inconclusive re TP expression and sensitivity to 5-FU, although it could be hypothesised that increased expression would relate to sensitivity. [86] [87] [3]

If a patient is administered capecitabine instead of 5-FU then the two proteins involved in the conversion to 5-FU are cytidine deaminase (CDA) and thymidine phosphorylase (TP). CDA is responsible for the catabolism of 5-deoxyfluorocytidine (5-DFCR) to 5-deoxyfluorouridine (5-DFUR).

This is the second step in the conversion of capecitabine to 5-FU.

Thymidylate synthase (TS) expression levels in colorectal cancer have been researched extensively as a single predictor of 5-FU resistance. Research has shown that low expression is linked to 5-FU sensitivity and also that amplification is linked to 5-FU resistance. [11] [88] Low TS expression has been significantly linked with 5-FU sensitivity in females. TS is phosphorylated to thymidine triphosphate which plays a key role in DNA synthesis and repair. 3 [89] [90] 5-fluorodeoxyuracil monophosphate (5-FDUMP) suppresses TS by forming a covalent ternary complex with 5, 10-methylenetetrahydrofolate, and thus indirectly affects DNA synthesis. This makes TS an ideal target for chemotherapeutic drugs.

RRM1 and RRM2 expression levels have been investigated both pre-clinically and clinically, and decreased levels have been linked with resistance to 5-FU. [90]

Both ribonucleotide reductases have been quantified in colorectal tumour versus non-tumour and it was hypothesised that increased expression in the tumour cells is linked with lymph node metastases.[91]

Human equilibrative nucleoside transporter 1 (hENT1) is a transmembrane protein responsible for the transport of gemcitabine into the cell. Recent research has shown that hENT1 expression in tumour samples of colorectal cancer was indicative of the clinical response to 5-FU - high hENT1 expression indicated a poor response to 5-FU. This was reversed when hENT1 was inhibited chemically in a colorectal cancer cell line.[92]

Research has been undertaken to compare protein expression in colorectal primary tumours with the corresponding liver metastases. This has indicated that expression

increases in the liver metastases and this should be considered when prescribing 5-FU. [93]

To summarise, resistance to 5-FU in colorectal cancer may be predicted using a combination of the following:

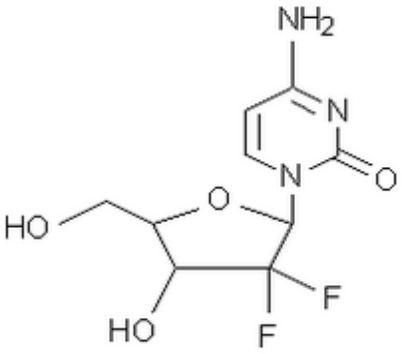
PROTEIN	EXPRESSION	
TS	Increased	Could be predictive of 5-FU resistance
DPYD	Increased	
TP	Inconclusive	
RRM1	Decreased	
RRM2	Decreased	
CDA	Decreased	
hENT1	Increased	

Table 1.7 Protein expression which may be predictive of resistance to 5-FU

1.4.2 Gemcitabine

Gemcitabine is a nucleoside analogue used in the treatment of many solid tumours, including pancreatic cancer. Until around 1997, 5-FU was the standard chemotherapy drug until it was found that gemcitabine conferred significantly increased survival times. Since then gemcitabine has been the first line drug for local advanced and metastatic pancreatic cancer. [94]

1.4.2.1 Structure and metabolism of gemcitabine



Gemcitabine

Gemcitabine is a an analogue of Cytosine Arabinoside (Ara-C), the difference being fluorine components on position 2' of the furanose ring.
The metabolism of gemcitabine results in inhibition of DNA polymerase and incorporation into DNA thus resulting in apoptosis.

Figure 1.16 Structure of gemcitabine

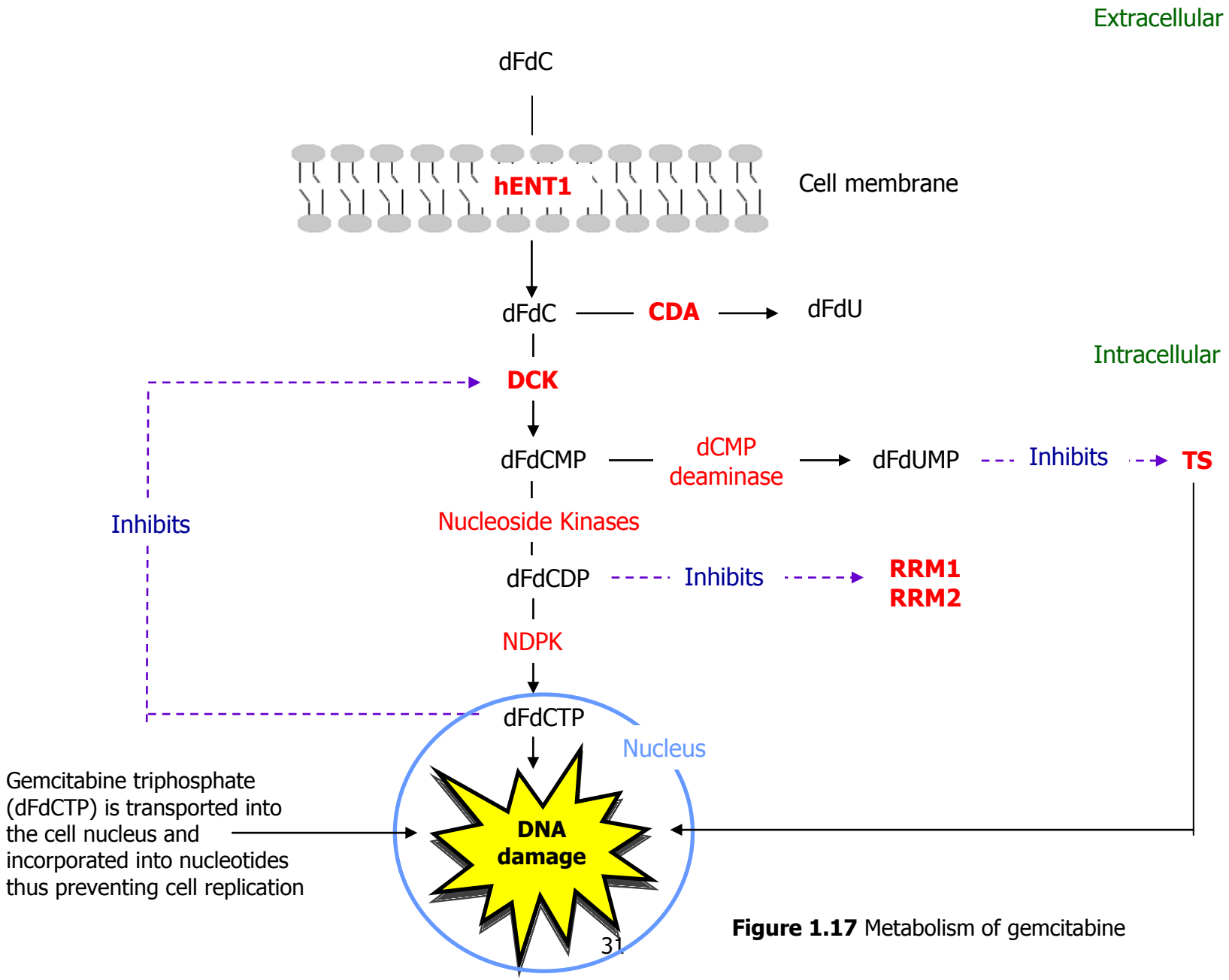


Figure 1.17 Metabolism of gemcitabine

Key:

dFdC – 2′, 2′ difluorodeoxycytidine or gemcitabine

hENT1 – human equilibrative nucleoside transporter

dFdCMP – 2′, 2′ difluorodeoxycytidine monophosphate or gemcitabine monophosphate

CDA – cytidine deaminase

DCK – deoxycytidine kinase

dFdCDP – 2′, 2′ difluorodeoxycytidine diphosphate or gemcitabine diphosphate

dFdU – 2′, 2′ difluorodeoxyuracil

dFdUMP – 2′, 2′ difluorodeoxyuracil monophosphate

dFdCTP – 2′, 2′ difluorodeoxycytidine triphosphate or gemcitabine triphosphate

dCMP deaminase – deoxycytidine monophosphate deaminase

RRM1 – ribonucleotide reductase subunit M1

RRM2 – ribonucleotide reductase subunit M2

NDPK – nucleoside diphosphate kinase

TS – thymidylate synth(et)ase

Figure 1.17 shows the conversion of gemcitabine to its active state, dFdCTP. Gemcitabine is hydrophilic and cannot traverse cell membranes by passive diffusion. Therefore, the presence and activity of human equilibrative nucleoside transporter 1 (hENT1) is considered a possible important determinant of gemcitabine cytotoxicity and clinical efficacy. Ninety per cent of gemcitabine is inactivated to dFdU (difluorodeoxyuridine) by cytidine deaminase (CDA), therefore reduced levels of CDA could correlate with increased gemcitabine activity. Intracellular gemcitabine (dFdC) is phosphorylated by deoxycytidine kinase (DCK) in a rate-limiting step to gemcitabine monophosphate (dFdCMP) – DCK is inhibited by dFdCTP.
RRM1 and RRM2 provide the precursors necessary for DNA synthesis. They are responsible for catalysing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides.

1.4.2.2 Gemcitabine resistance in cancer

Research has demonstrated that acquired and inherent chemoresistance of pancreatic cancer cells to gemcitabine is determined by the balance of DCK, RRM1, RRM2, and hENT1 gene expression, but not to that of any of the individual genes. The hENT1*DCK/RRM1*RRM2 expression ratio significantly correlates with resistance to gemcitabine in pancreatic cancer cells, including acquired gemcitabine-resistant cells, suggesting that a decrease of this ratio reflects inherent and acquired chemoresistance

of pancreatic cancer cells to gemcitabine and may be a key to understanding the variable effectiveness of gemcitabine among individual patients. However, this same study found hENT1 expression levels did not change in resistance to gemcitabine. [95]
Lecca et al conducted a review of gemcitabine resistance and generated an almost identical formula - DCK/RRM1*RRM2 with the hypothesis that hENT1 is an independent indicator of resistance to gemcitabine. [128]
hENT1 protein expression was shown to be associated with increased overall survival and disease-free survival in pancreatic cancer patients who received gemcitabine, but not in those who received 5-FU. These results could indicate that hENT1 expression could possibly be a predictor of gemcitabine sensitivity in patients with pancreatic cancer. [96]
hENT1 expression has been shown to be an independent factor associated with survival in patients with pancreatic cancer; however hENT1 expression might be a possible new prognostic factor for chemosensitivity of pancreatic cancer to gemcitabine. [97]
Quantitative analysis of hENT1, DCK, RRM1, and RRM2 mRNA using FFPE tissue samples and evaluation of a combined gemcitabine score were deemed useful in predicting the sensitivity to gemcitabine-based adjuvant chemotherapy in patients with pancreatic ductal adenocarcinoma (PDAC). Also, quantitative analysis of these genes in tumour cells taken from fine needle aspiration (FNA) specimens was found useful in determining the treatment for patients with PDAC even when the tumour is unresectable. [98]
Thymidylate synth(et)ase (TS) is possibly the most researched protein in 5-FU resistance. It is inhibited by dFdUMP which would result in increased activity of hENT1, which is inhibited by TS. This is highlighted by the fact that TS inhibitors allow increased expression of hENT1, therefore TS expression could be indirectly involved in gemcitabine resistance.
hENT1 is a transmembrane protein. In order for transmembrane proteins to localise to the plasma membrane, they must have been processed without any errors. Research has shown that hENT2 (a transmembrane protein also involved in the transport of gemcitabine across the membrane) can undergo disrupted localisation which results in resistance to gemcitabine despite being expressed. [99] Other research has shown that disruption of a triplet near the N-terminus, or the last eight C-terminal residues result in loss of plasma membrane localisation and/or transport function of hENT1. [100]

To summarise, resistance to gemcitabine in pancreatic cancer may be predicted using a combination of the following:

PROTEIN	EXPRESSION	
hENT1	Decreased	Could be predictive of gemcitabine resistance
CDA	Increased	
DCK	Decreased	
RRM1	Increased	
RRM2	Increased	
TS	Increased	

Table 1.8 Protein expression which may be predictive of resistance to gemcitabine

1.5 Tissue Microarrays

1.5.1 Tissue microarray (TMA) or whole sections

Tissue microarrays (TMAs) comprise of one paraffin block with 2-1000 tissue cores. TMAs are constructed by taking individual cores (0.5-2 mm in diameter) from FFPE blocks and placing them into a master block in a grid format.

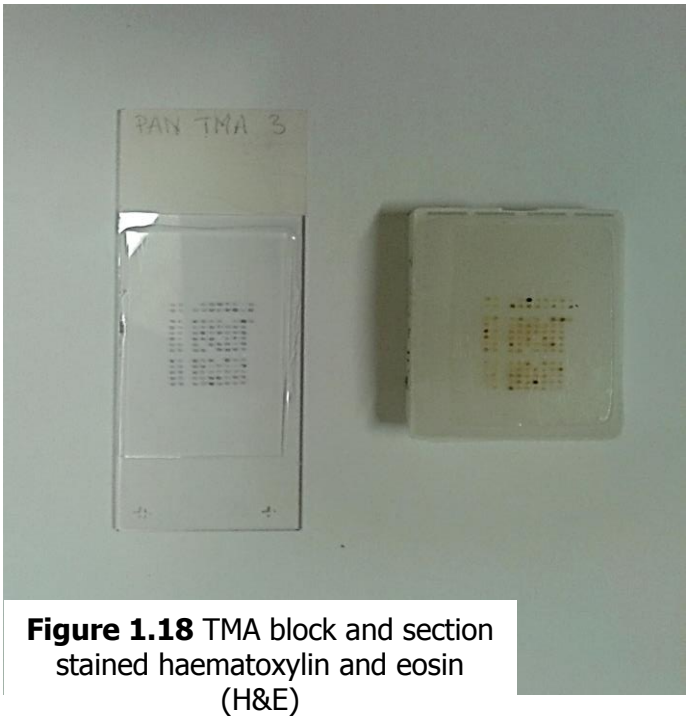


Figure 1.18 TMA block and section stained haematoxylin and eosin (H&E)

As early as 1998, the process describing the technique for high throughput profiling of tumour specimens was published. [101] Since then, the use of TMAs has grown enormously. [102] There are pros and cons for the technique - on the plus side, large amounts of different tissue cores may be embedded into one paraffin block. This not only preserves the original tissue section but saves money as the use of reagents is much diminished for TMAs. On the minus side, a lot of time is spent marking out tumour areas on stained slides of whole sections. This tends to be done by Biomedical Scientists or Research Assistants. There is the possibility that the tumour area may be misinterpreted and the incorrect area cored. This is mitigated by constructing the paraffin blocks in triplicate at the very least. This also allows for tumour heterogeneity, ideally different histological areas of the whole block are taken. Other research has shown that TMAs are equal to whole sections in heterogeneic tumour sections. [103] There is also the risk of losing cores from the TMA when sectioning. [104] Again this is mitigated by constructing multiple blocks.

To summarise, TMAs help preserve precious tissue samples, are very cost effective and allow high throughput of samples. [105] [101]

Figure 1.19 Semi-automatic arrayer

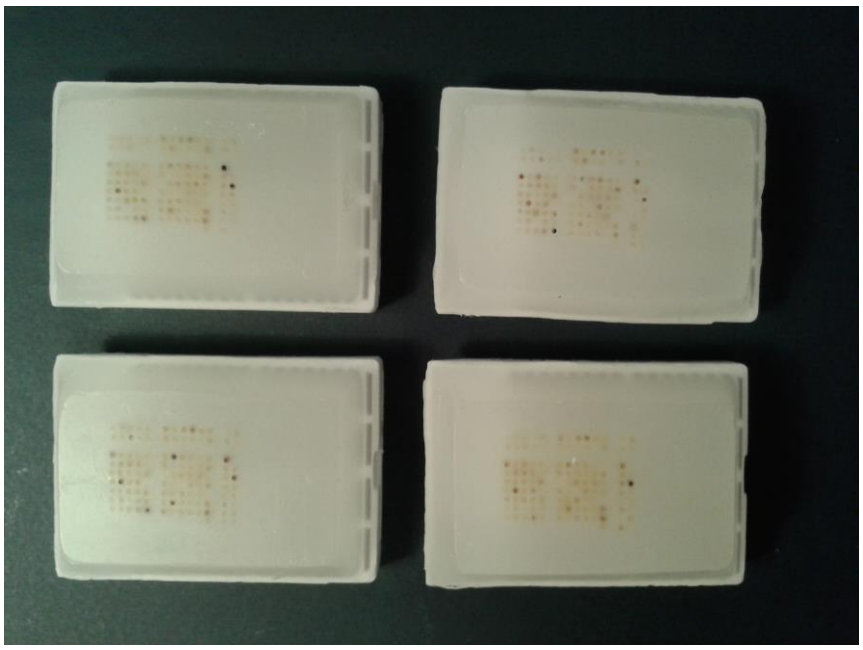


Figure 1.20 Set of 4 identical TMA blocks

1.5.2 Long term storage of TMA sections

The time and effort taken to produce TMAs means that serial sections are cut in a bid to maximise the amount of tissue available. These sections are invariably stored for future use and it is generally perceived that tightly wrapped sections stored at -20°C is the best method to preserve antigenicity. DiVito et al found that the optimum storage

for cut TMA sections was paraffin coating and storage in a nitrogen dehumidifier, although this was only assessed over a 3 month period. [106]

Karlsson and Karlsson compared the storage of TMA sections at room temperature, 4°C and -20°C. No significant difference was found in the staining intensity of IHC and fluorescent in-situ hybridisation over a period of 1 year, with storage at 4°C slightly superior to the other conditions. [107]

There could be several reasons for this – tissue being processed in CPA and other accredited labs is adequately fixed and processed. Small research labs cannot offer the same assurance. Antigen retrieval methods are improving over time, which would appear to negate the decline in antigenicity over a period of time. Also, the modern-day highly-sensitive antibody detection kits are far superior to historic ones.

These factors mean that TMA sections can be cut and stored for future use with the reassurance that DNA and RNA targets are preserved.

1.6 Automated QUantitative Analysis (AQUA)

1.6.1 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) or "brown staining" remains the standard practice for staining and quantifying protein expression in diagnostic labs worldwide. IHC utilises immunological, histological and biochemical techniques to identify specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. IHC makes it possible to visualise the localisation of specific cellular components within a cell or tissue. Nowadays, the focus for IHC has shifted from being used as a diagnostic aid to being used as a prognostic and predictive tool by pathologists worlwide. This has changed the goalpost from a simple positive or negative dignosis to the need for quantitative analysis with the subcellular localisation of the protein. There are many variables that may affect IHC staining and scoring:

1) Fixation and processing, thickness of sections
2) Storage medium of cut sections
3) Antigen retrieval system used
4) Staining method used
5) Dilution of antibidy used
6) Reagent validation
7) Experience of technician
8) Positive and negative controls used
9) Subjective nature of scoring [108]

Variables 1-8 can be addressed by implementing a quality control/assurance programme for all pre-clinical and clinical research, however the results will always remain subjective.

AQUA (automated quantitative analysis) utilises an immunofluoresecent technique to identify and visualise the target protein. The amount of protein expressed is calculated by dividing the intensity of fluorescence by the total area of tumour tissue scanned. The use of AQUA has been shown to be superior to and remove the subjectivity of IHC scoring by pathologists, which can only be described as semi-quantitative at best. It is of vital importance that IHC is scored accurately as any discrepancies could lead to patients being misdiagnosed and even being prescribed the wrong treatment regimen. [109] [110]

1.6.2 Automated Quantitative Analysis (AQUA)

Because of the subjectivity of IHC scoring there are a number of computer-based quantitative analysis packages commercially available.

The AQUA system was first developed in the Department of Pathology at Yale University. [111]

Normally the pathologist will look at sections and score them – perhaps even using a written method in a bid to standardise the process – by looking at staining intensity and number of positive cells. This method is neither accurate nor reproducible, the same pathologist can score the same section differently if it is done at different times. [112] [108]

AQUA utilises immunofluorescent (IF) based IHC rather than the conventional chromogenic staining to visualise protein expression. Firstly, a cytokeratin mask is applied to the section to enable differentiation between tumour cells and cell stroma. Then antibody-conjugated fluoresecent dye is used to stain the target protein, instead of the diaminobenzidine (DAB)/immunoperoxidase (or brown staining) used in conventional IHC. Finally, the a nuclear counterstain is used, which is 4,6-Diamidino-2-phenylindole (DAPI) for AQUA instead of haematoxylin which is used in conventional IHC.

Camp et al devised a series of algorithms that he called AQUA which allow rapid, high-throughput analysis of TMAs.

The first algorithm, called PLACE (Pixel based Locale Assignment for Compartmentalisation of Expression), separates tumour cells from the stroma and defines the subcellular compartments using fluorescent labels. The target protein is then quantified and located using these fluorescent tags. The amount of protein

expressed is calculated by dividing the intensity of fluorescence by the total area of tumour tissue scanned.

The second algorithm, called RESA (Rapid Exponential Subtraction Algorithm), allows for analysis of overlapping subcellular compartments, despite the thickness of the tissue sections, by subtracting an out-of-focus image from a focussed one based on pixel intensity, signal-to-noise ratio, and the expected compartment size. [111]

These algorithms were validated using oestrogen receptor positive (ER positive) breast cancer tissue and colon carcinoma comparing pathologist scoring with AQUA quantification.

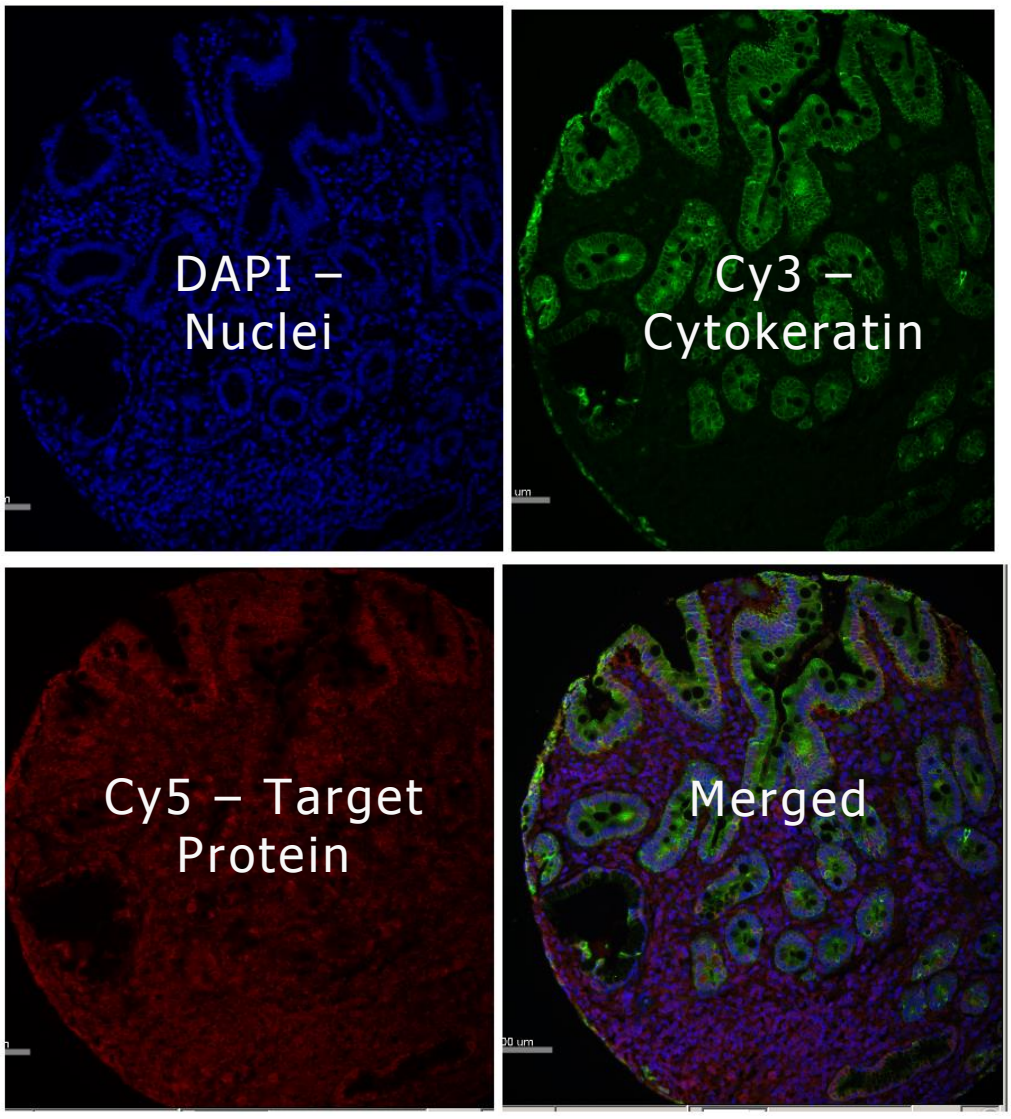


Figure 1.21 Pancreatic TMA core showing
1) DAPI-stained nuclei
2) Cytokeratin mask
3) Target protein
4) All three

1.7 Aims and Objectives

1.7.1 Quality

This thesis aims to address the reported factors that may contribute to academic research results irreproducibility. The constraints are the same for all academic researchers – lack of time and funds. However, even with the limitations stated above, it is believed that this thesis is reproducible and transparent. All quality measures taken for this project are described in Chapter 2.

The primary objective of the quality aspect of the project is to produce and document the quality control/quality assurance actions implemented to ensure accuracy and reproducibility.

1.7.2 5-FU and capecitabine resistance

Despite major advances in research over the past 50 years, drug resistance still remains the major obstacle in many cancers.

5-FU only has a response rate of 10-15% in advanced colorectal cancer, which increases to 40-50% when used in combination with other drugs.[113]

This thesis aims to research and identify the mechanisms of resistance to 5-FU (and capecitabine) thus enabling oncologists to accurately predict who will benefit from these therapies and negate the need for unnecessary treatment. The proteins involved in transforming capecitabine to 5-FU are CDA and TP. [114] The proteins involved in the metabolism of 5-FU to its active state are TS, DPYD, TP, RRM1, RRM2, CDA and hENT1. [3 88 90 92]

Three sets of TMAs have been constructed and will be utilised in this thesis – colorectal cancer all stages, colorectal primary cancer with matched liver metastases. These will be analysed using AQUA to determine subcellular location and quantification of expression levels of the above proteins within tumour cells. This will be correlated against various demographics including chemotherapy regimen received and overall survival time.

The primary objective of this part of the project is to discover the proteins and the relationship (if any) between the proteins, which will predict response to these drugs.

1.7.3 Gemcitabine Resistance

Resistance to gemcitabine, either inherent or acquired, is a major problem for oncologists. [115] The clinical response to gemcitabine is less than 25%, and most tumours that initially respond will develop resistance. This could be due to further mutations within the tumour cells or the heterogeneity of the existing tumour, which

allows a subset of the cells to continue to grow. Due to the late diagnosis of pancreatic cancer, only 50% of patients diagnosed with this cancer are alive after 6 months and the 5-year survival rate is less than 5%. [116]

These statistics are horrifying and because survival times are so short it is paramount that patients are given treatments that are effective. This thesis will endeavour to identify the combination of proteins involved in resistance. The proteins involved in the transport into the cell and conversion of gemcitabine to its active state are hENT1, CDA, DCK, RRM1 and RRM2. [78 95 98] One set of quadruplicate TMAs has been constructed of different grades of pancreatic cancer, and protein expression and subcellular location will be determined using AQUA analysis.

This will be correlated against patient demographics including chemotherapy regimen received and overall survival time.

The primary objective of this part of the project is to discover the proteins and the relationship (if any) between the proteins, which will predict response to gemcitabine.

2. Quality, Materials and Methods

2.1 Quality

2.1.1 Quality Management

Quality management encompasses both quality control and quality assurance.

Quality management is a means of ensuring that a product or process attains the required level of quality. Depending on the circumstances, the level of quality could be determined by either regulatory requirement or adherence to local requirements. This project utilised both quality control (QC) and quality assurance (QA) to ensure that all steps taken were transparent and reproducible. This project utilised archival clinical material, therefore the standards aimed to be measured against were Good Clinical Practice (GCP) and Good Laboratory Practice (GLP).

Emphasis was placed on the areas of study which could potentially influence the results wherever possible. A flowchart of study processes/ procedures and table of predicted risks and risk mitigation, to ensure accurate and reproducible results, is detailed below. A work plan was produced prior to starting any lab work, and this can be seen in Appendix 3.

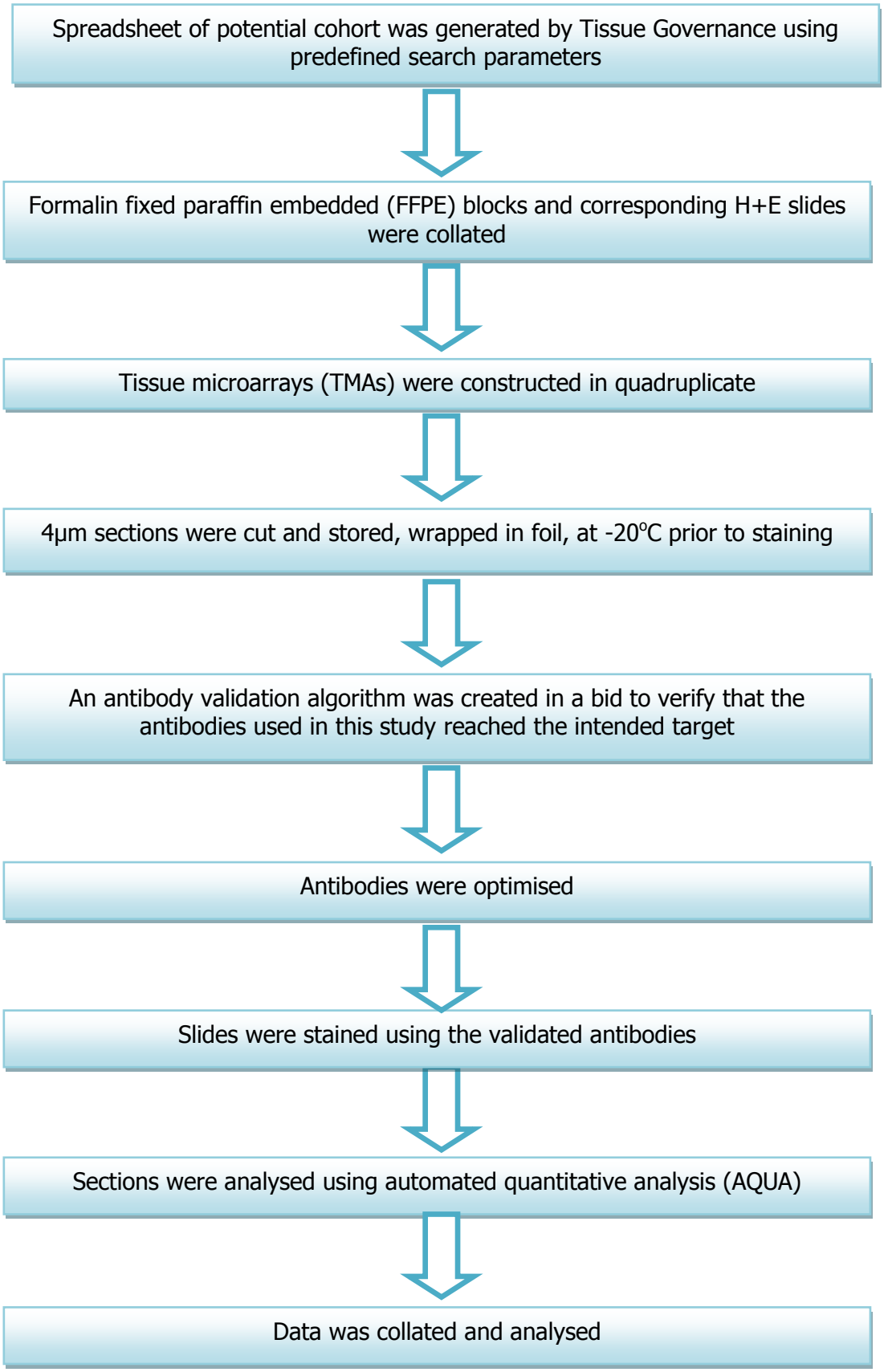


Figure 2.1 Flowchart to show the order of work for this project

The figure above shows a brief overview of the work undertaken and the order it was completed in. This is explained in more detail in the methods section of this chapter. A QA programme was conducted throughout the duration of the study and a final QA report detailing the audits undertaken and any findings.

Stage of project	Possible risk	Mitigation
Spreadsheet of potential blocks	Blocks contain no or inadequate tumour tissue	All blocks were checked to ensure sufficient tumour tissue was present
FFPE blocks and H+E slides collated	Incorrect slide collected and marked out	All blocks and slides were reconciled
TMAs constructed	Tumour area missed	TMAs were constructed in quadruplicate
TMAs constructed	Transcription error on TMA map	All TMA maps were reconciled against blocks and slides and QC checked by 2 people
4µm sections cut and stored	Storage conditions affect antigenicity	Extra slides were cut and stored under different conditions prior to staining to ascertain optimum storage conditions were used
Antibody validation algorithm created prior to any staining	Antibodies not specific to target protein	Western blots to see if band appeared at predicted molecular weight (MW)
Antibody validation algorithm created prior to any staining	Antibodies not specific to target protein	Stained sections checked for cleanliness and target area of cell stained
Antibody validation algorithm created prior to any staining	Antibodies not specific to target protein	Antibodypedia consulted to check if antibody validated by other researcher
Slides stained	Error whilst staining	QA programme instigated and inspections carried out by an independent QA manager
Section analysis using AQUA on 2 different pieces of equipment	No correlation between results	Results were compared and analysed to ensure correlation of results
Data collection and analysis	Error in data collation	Data audit conducted by independent QA manager
Data collection and analysis	Data manipulated to enhance results	Final study audit conducted by independent QA manager

Table 2.1 Table of anticipated risks and mitigations. These will be explained in greater detail in the methods section of this chapter. This list is not exhaustive but an example of the measures that may be taken by an investigator with limited time and funds.

2.1.1.1 Good clinical laboratory practice

GCP is a set of guidelines which is primarily concerned with the rights and well-being of participants taking part in clinical trials, although there is a large section on the validity of data.[117]

GLP is a set of standards applied to pre-clinical studies.[118] Pre-clinical means laboratory work that involves animals or cell lines, not human samples. The guidelines are focussed on the generation and validity of results, to ensure that the results produced are reliable and reproducible.

Good clinical laboratory practice (GCLP) is a merging of these two sets of standards, thereby ensuring that the work being done in the lab is conducted to GLP standards whilst ensuring the rights and well-being of study participants is not compromised.[119] This study focussed on ensuring patient confidentiality as well as the generation, validation and interpretation of results. All steps taken to ensure this are detailed in this chapter.

2.2 Materials

2.2.1 TMA set 1 – Colorectal cancer TMA

A cohort of patients was generated from the NHS Lothian Pathology Archive Database using the search terms "colorectal", "adenocarcinoma" and "resection". A list was finalised which included both males and females of varying ages and all stages of colorectal cancer, who had undergone a resection procedure. Ethical approval was granted by Scotland A REC (10/S1402/33) for the generic use of pathology archive tissue for research. An abbreviated table of patient characteristics can be seen in table 2.2 below. A more detailed table of patient characteristics can be seen in Appendix 4.

Characteristic	Number of patients	Per cent %
Sex	**130**	**100**
Male	70	53.8
Female	60	46.2
Age (years)	**130**	**100**
<50	8	6.2
≥50	122	93.8
Duke's stage	**130**	**100**
A	12	9.2
B	45	34.6
C	67	51.6
D	3	2.3
NK	3	2.3
Maximum tumour diameter (mm)	**130**	**100**
<25	12	9.2
≥25 and <50	74	57
≥50 and >100	36	27.7
>100	5	3.8
NK	3	2.3
Tumour location	**130**	**100**
Colon	110	84.6
Rectum	19	14.6
NK	1	0.8
Tumour differentiation	**130**	**100**
Moderate	104	80
Moderate/poor	6	4.7
Poor	15	11.5
Well	3	2.3
NK	2	1.5

Table 2.2 Patient characteristics for patients with colorectal adenocarcinoma included in this study that underwent colonic resection

The above 130 blocks were used to construct TMAs in quadruplicate which were cut and stained prior to AQUA analysis. The proteins stained immunofluorescently were TP, RRM1, RRM2, DPYD and TS. The analysis was run using TMA Navigator, a

statistical programme which automatically divides the cohort into three equal groups.

Univariate analysis was performed to produce overall and disease free survival. The analyses were conducted on:

- All patients
- Patients who received gemcitabine
- Patients who did not receive any chemotherapy

2.2.2 TMA set 2/3 – Colorectal cancer/matched liver metastases TMA

A cohort of patients was generated from the NHS Lothian Pathology Archive Database using the search terms "liver", "adenocarcinoma", "metastatic" and "colorectal". A list was finalised which included both males and females of varying ages with a primary diagnosis of colorectal cancer and confirmed liver metastases. Ethical approval was granted by Scotland A REC (10/S1402/33) for the generic use of pathology archive tissue for research. An abbreviated table of patient characteristics can be seen in table 2.3 below. A more detailed table of patient characteristics can be seen in Appendix 5.

Characteristic	Number of patients	Per cent %
Sex	**68**	**100**
Male	40	59
Female	28	41
Age (years)	**68**	**100**
<50	3	4
≥50	65	96
Maximum tumour diameter (mm)	**68**	**100**
<25	10	15
≥25 and <50	39	57.5
≥50 and >100	13	19
>100	2	3
NK	4	5.5
Tumour location	**68**	**100**
Colon	40	58.5
Rectum	27	40
NK	1	1.5
Tumour differentiation	**68**	**100**
Moderate	56	82.5
Moderate/poor	2	3
Poor	7	10
Well	2	3
NK	1	1.5

Table 2.3 Patient characteristics for patients included in this study who had a primary colorectal tumour and metastatic liver tumour(s)

2.2.3 TMA set 4 – Pancreatic cancer TMA

A cohort of patients was generated using the NHS Lothian Pathology Archive Database using the search terms "pancreas", "adenocarcinoma" and "Whipple's resection". A list was finalised which included both males and females of varying ages and stages of pancreatic cancer. Ethical approval was granted by Scotland A REC (10/S1402/33) for the generic use of pathology archive tissue for research. An abbreviated table of patient characteristics can be seen in table 2.4 below. A more detailed table of patient characteristics can be seen in Appendix 6. Authorisation from Lothian Tissue Governance to retrieve archival FFPE blocks can be seen in Appendix 7.

Characteristic	Number of patients	Per cent %
Sex	**120**	**100**
Male	66	55
Female	54	45
Age (years)	**120**	**100**
<50	8	6.7
≥50	112	93.3
Maximum tumour diameter (mm)	**120**	**100**
<25	21	17.5
≥25 and <50	88	73.35
≥50 and >100	10	8.35
>100	0	0
NK	1	0.8
Tumour location	**120**	**100**
Head of pancreas	82	68.35
Body of pancreas	3	2.5
Tail of pancreas	4	3.35
Other	31	25.8
Tumour differentiation	**120**	**100**
Moderate	50	41.7
Moderate/poor	12	10.0
Poor	41	34.2
Well	16	13.3
NK	1	0.8

Table 2.4 Patient characteristics for patients with a diagnosis of pancreatic adenocarcinoma and underwent a pancreatic resection

The above 120 blocks were used to construct TMAs in quadruplicate which were cut and stained prior to AQUA analysis. The proteins stained immunofluorescently were hENT1, DCK, RRM1, RRM2. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups.

hENT1 – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were

undertaken to see if the two methods of analysis produced conflicting results. hENT1 cytoplasmic expression was calculated using AQUA for both median and combat values.

Lecca formula – this was used for multivariate analysis of the proteins involved in gemcitabine metabolism. Details of the Lecca formula can be found in section 2.3.5. Both univariate and multivariate analysis were performed to produce overall and disease free survival. The analyses were conducted on:

- All patients
- Patients who received gemcitabine
- Patients who did not receive any chemotherapy

2.3 Methods

2.3.1 Tissue microarray

Sections were cut from each donor block at 4µm using a Leica rotary microtome, model no. RM2235 and stained with haematoxylin and eosin (H+E): this stains the nuclei blue and the cytoplasm pink. Briefly, sections were dewaxed in 3 changes of xylene for 5 minutes each. Slides were then rehydrated through a series of alcohols – 100%, 100%, 80% and 50% - 2 minutes in each, then washed in running water for 2 minutes.

Slides were stained in Harris haematoxylin (supplied by Shandon) for 4 minutes and washed in running water for 2 minutes. Slides were then placed in Scott's tap water substitute until the sections turned blue. The nuclei were checked microscopically to ensure the correct level of staining. If too dark the slides were differentiated in acid/alcohol, washed in water and blued in Scott's tap water substitute. If too light they were placed in haematoxylin for a further 30 seconds and washed and blued as previously described. Once the correct level of nuclear staining was achieved the slides were washed in running water for 2 minutes. Slides were then stained in eosin (eosin Y aqueous stain supplied by Shandon) for 5 minutes and washed in running water for 1 minute. The slide rack was allowed to drain well and slides dehydrated through alcohols – 50%, 80%, 100% and 100% - for 30 seconds, 30 seconds, 2 minutes, 2 minutes. Slides were cleared in 3 changes of xylene, 5 minutes each and mounted using DPX. See Appendix 8 for SOP of staining method used.

The slides were then examined microscopically and tumour areas marked out. Tumour areas that appeared different histologically were taken in an attempt to ensure that tumour heterogeneity was taken into account. It is logical to hypothesise that tumour which appears different under the microscope may possess different biological properties. See Appendix 9 for SOP referred to during marking out the slides.

Four 0.6mm cores were taken from each block and arrayed in quadruplicate in recipient paraffin wax blocks. A manual tissue arrayer (Beecher, model MTA1) was used to construct the TMAs. Histologically different areas were taken (where possible) to see if different tissue morphology corresponds to different expression of target proteins.

This is covered in the results chapter by analysing median values of TMAs as well as all values.

Figure 2.2 H+E stained slide.
This has been marked out by a pathologist, the black circles are areas of tumour and 1/2 means that 1 out of 2 lymph nodes present on the slide contains metastatic adenocarcinoma. The block to the right hand side of the slide is the tumour block the section on the slide came from.

Slides and blocks were reconciled to ensure the correct slide had been marked out. 4µm sections were cut from each TMA on a Leica rotary microscope and sections stored, separated by tissue and wrapped in foil in an airtight box, in a -20°C freezer.

2.3.1.1 Quality control of TMAs and unstained slide storage conditions

QC of TMAs

All TMAs were constructed in quadruplicate. During construction all slides/blocks were placed in order in a storage box. The slides/blocks were reconciled against the TMA map by the researcher and an independent person. Both persons then signed the TMA map as witness that the blocks were cored and recorded in the correct order. Any discrepancies were noted and corrected.
See Appendix 10 for an example of a TMA map.

Unstained slide storage conditions

3 unstained slides were selected from the same TMA and stored for 6 months at room temperature (RT), 4°C and -20°C.

These sections were then stained using the method described in section 2.3.3 IF staining, using a rabbit primary antibody against TP and the staining compared on all 3 slides.

The sections for this study were stored at -20^{o}C however, research has shown that the storage temperature is not critical to retention of antigenicity and that storage at 4^{o}C is slightly better. [120]

2.3.2 Antibody validation

Antibody centred techniques such as Western blotting, enzyme-linked immunosorbent assay (ELISA), immunoprecipitation (IP), immunofluoresence (IF), immunohistochemistry (IHC) and reverse phase protein microarray (RPPA) are used in both clinical and research settings however, antibody validation is seldom implemented to ensure the quality, reproducibility and specificity of the antibodies. The reasons for this could include lack of resources and time, as well as the poor quality of commercially available antibodies. Can we rely on the validity and specificity of antibodies currently employed in preclinical research and have confidence in the reliability of the data found in peer-reviewed publications? [121]

IHC is routinely used in the clinical setting, the results of which inform the clinician which treatment is best for the patient. Antibody based procedures used in a research laboratory could ultimately be a source of direction for large pharmaceutical companies.

The Human Protein Atlas (HPA) is a research project that aims to map out the human proteome using an antibody based approach. Initiated in 2003, this project systematically generates and validates antibodies, using expression profiling against a range of normal tissues, cancer tissues and cell lines. [122] The information in the table below was taken from the HPA.

ANTIBODY	VALIDATION LEVEL IN HPA	EXPRESSED	COMMENTS
hENT1	Uncertain	Nucleus/Cytoplasm/ Membrane	Low to high expression in colorectal cancer and low to medium expression in pancreatic cancer
TS	Uncertain	Cytoplasm/Membrane	Medium to high expression on colorectal cancer and low to medium expression in pancreatic cancer
DPYD	Uncertain	Cytoplasm/Membrane	No expression in colorectal cancer and low expression in pancreatic cancer
TP	Supportive	Nucleus/Cytoplasm	Low to high expression in colorectal cancer and low to medium expression in pancreatic cancer
RRM1	Uncertain	Cytoplasm/Membrane	Low to high expression in colorectal cancer and low to medium expression in pancreatic cancer
RRM2	Supportive	Cytoplasm/Membrane	Low to medium expression on colorectal cancer and low expression in pancreatic cancer
DCK	No details in HPA	No details in HPA	No details in HPA
CDA	No details in HPA	No details in HPA	No details in HPA

Table 2.5 A list of all antibodies used in this study and the level of validation achieved in the Human Protein Atlas.

Only TP and RRM2 could be considered validated using the HPA. Therefore, this project attempted to validate all antibodies prior to any staining being undertaken. [123] An antibody validation algorithm was developed, which could be easily integrated into a research or clinical laboratory – see below. It is anticipated that the process of antibody validation coupled with the results from this project will validate the antibody validation algorithm.

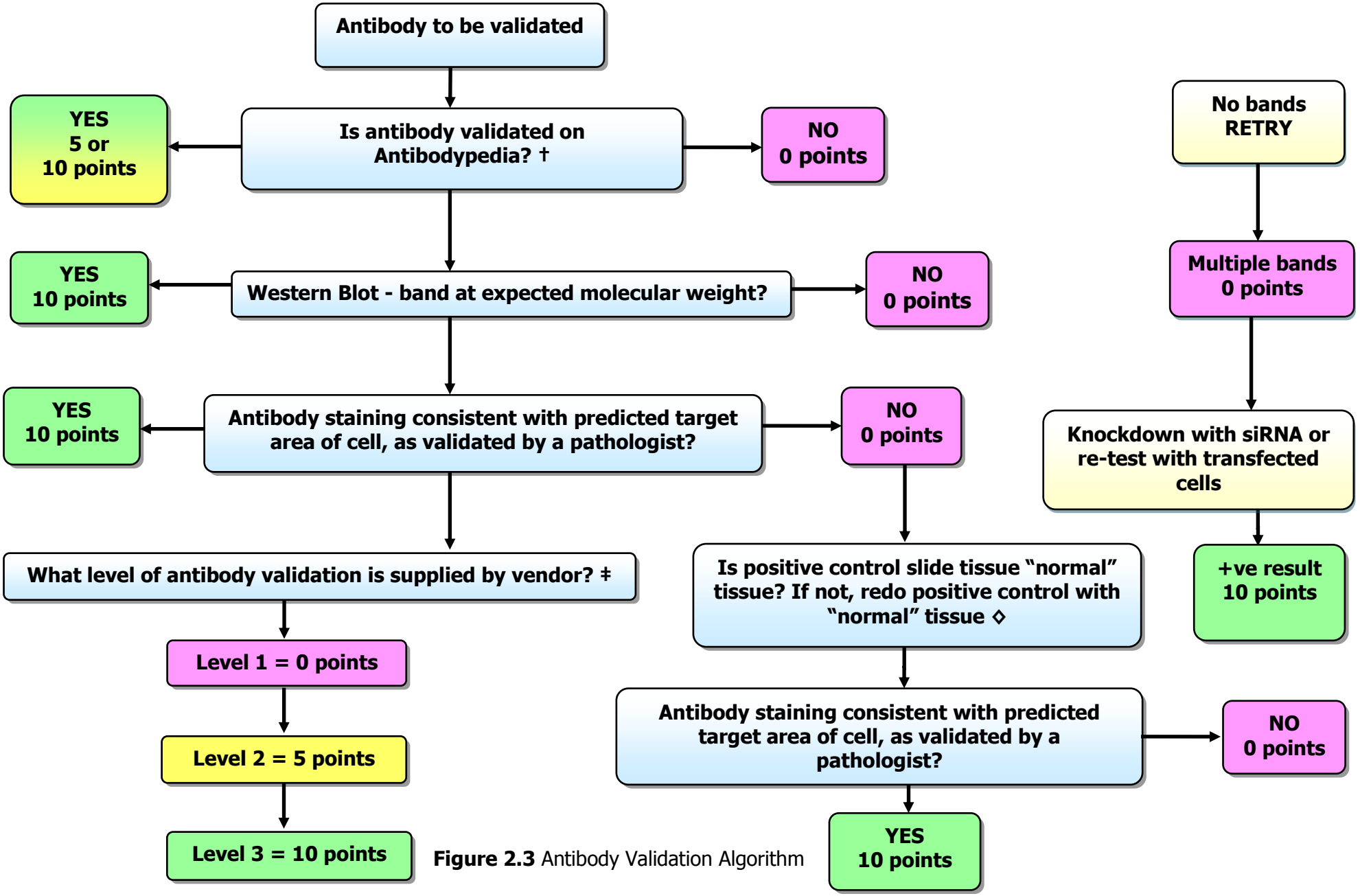


Figure 2.3 Antibody Validation Algorithm

2.3.2.1 Rationale

The above antibody validation algorithm was created in a bid to at least attempt to verify that the antibodies used for this study were specific to the target protein. Inspiration was taken from the CRUK prognostic/predictive biomarker roadmap, the human protein atlas and literature review. The purchase of antibodies was capped at 3 due to cost.

Out of a potential 45 points, each antibody must score at least 30 points to be considered validated for use in research. This algorithm is not exhaustive; however it is a method of validation for use in small research facilities that have no formal processes in place for antibody validation and limited funds.

† Antibodypedia www.antibodypedia.com

This is an open-access database of commercially available antibodies against human protein targets. The site provides information on the efficacy of antibodies within a range of techniques. There is no subscription charge, the information available is free. Researchers submit their data generated for an antibody which will either validate or invalidate the efficacy of the antibody. Details will include the supplier of the antibody, protocol used and application performed: Western blot, IHC, IF or FISH. Staining images are also submitted and researchers are able to leave comments. Fulfilment of all these points will score 10 points. [124]

If there are no details for the particular antibody but details are present for the same antibody from the same supplier used for a different application – score 5 points.

‡ Levels of validation by antibody vendor

Level 1

- very limited information on validation of antibody
- brief background information on target with very few references
- information is available on recommended applications and starting dilutions
- no examples of successful use in these applications
- no references from publications
- Datasheet contains information on animal host and immunogen source – i.e. whether it is a synthetic peptide or a purified protein
- no information on the peptide sequence

This level of validation scores 0 points on the algorithm therefore there are no minimum criteria points to be met.

Level 2

As level 1 validation criteria with the following included:

- no in-depth information on procedures for antibody validation
- datasheet contains background information on target and immunogen source
- Information is also available on the complete peptide sequence or the area surrounding the phosphorylation site
- Information is available on recommended applications and starting dilutions
- at least one example of the antibody successfully reaching the appropriate target in one of the recommended applications, e.g. a successful Western blot on cells which express the target

This level of validation scores 5 points on the algorithm. A minimum of 3 out of 5 criteria points must be reached to score 5 points.

Level 3

As levels 1 and 2 validation criteria with the following included:

- contains a description of the validation procedures employed by the vendor, e.g. a Western blot in multiple cell lines
- details on the quality control measures implemented to ensure no variations within different batches
- stringent antibody validation protocol which includes different applications on cell lines which express the target
- Information is present on optimal dilutions and preferred buffer

This level of validation scores 10 points on the algorithm. A minimum of 2 out of 4 criteria points must be reached to score 10 points.

◇ *Is positive control "normal" tissue, if not redo*

If the positive control tissue for an antibody is not "normal" tissue, it is renal carcinoma or epithelial ovarian cancer, then there is a chance that the target has been translocated within the cell due to either the disease or treatment of the disease. Therefore, if the epitope is clearly stained but not in the predicted area of the cell, it is reasonable to presume that the antibody has correctly identified the target protein. The target protein has changed location due to the underlying disease or the treatment

received. An alternative positive control should be sought using "normal" tissue and the two sections compared and analysed by a pathologist.

2.3.2.2 Antibody quality

The above algorithm was applied to all antibodies purchased for this project.
If the antibody failed to meet standards then the vendor was contacted and supplied with details. See Appendix 11 for the standard email sent to antibody suppliers. This process was continued until all antibodies were validated using the algorithm.
The table below details some of the antibodies purchased and the response from the vendors whilst in the process of finalising antibodies to be used.

ANTIBODY	SUPPLIER	+VE CONTROL	WESTERN BLOT	COMMENTS	SUPPLIER RESPONSE
hENT1 Human Equilibrative Nucleoside Transporter	Novus Biologicals Rabbit polyclonal Ab, NBP1-50508	Spleen Kidney	Fail (no expression)	To be redone if we can find a cell line which overexpresses hENT1	Full refund received from supplier with no comments.
hENT1 Human Equilibrative Nucleoside Transporter	Epitomics Rabbit polyclonal Ab, ab135756	Spleen Kidney	Fail (multiple bands)		Full refund received from supplier with no comments.
hENT1 Human Equilibrative Nucleoside Transporter	Proteintech Rabbit polyclonal Ab, 11337-1-AP	Spleen Kidney	Fail (multiple bands)		Full refund received from supplier with apology, no explanation.
CDA Cytidine Deaminase	Novus Biologicals Rabbit polyclonal Ab, NBP1-31412	Spleen	Fail (multiple bands)		Full refund received from supplier with no comments.
CDA Cytidine Deaminase	Epitomics Rabbit polyclonal Ab, Ab82347	Spleen	Fail (multiple bands)	2 bands - strongest at wrong MW	Full refund received from supplier with no comments.

ANTIBODY	SUPPLIER	+VE CONTROL	WESTERN BLOT	COMMENTS	SUPPLIER RESPONSE
CDA Cytidine Deaminase	Abnova Rabbit polyclonal Ab, NBP1-31412	Spleen	Fail (multiple bands)		CDA transfected 293T lysate received as compensation along with refund.
DCK Deoxycytidine Kinase	Lifespan Biosciences Rabbit polyclonal, LS-B1825 Rabbit polyclonal Ab	Pancreas	Pass		Supplier not contacted
TS Thymidylate Synth(et)ase	Abcam Rabbit mAb, ab108995	Colon carcinoma	Pass		Supplier not contacted
RRM1 Ribonucleotide Reductase M1	Proteintech Rabbit polyclonal Ab, 10526-1-AP	Spleen kidney	Pass		Supplier not contacted
RRM2 Ribonucleotide Reductase M2	Abcam Mouse mAb, ab115701	Spleen kidney	Fail	Faulty batch diagnosed	Replacement antibody received with explanation that previous antibody was from a faulty batch
DPYD Dihydropyrimidine Dehydrogenase	Cell Signaling Rabbit mAb, #4654	Liver carcinoma	Pass		Supplier not contacted
TP Thymidine Phosphorylase	Cell Signaling Rabbit mAb, #4307	Primary breast cancer	Pass		Supplier not contacted

Table 2.6 Antibodies bought and vendor response

2.3.2.3 Western Blot

Preparation of protein lysates and determination of protein content

Protein lysate samples were prepared and stored at -80°C. To summarise briefly, cell cultures were seeded into 14 cm (diameter) plates until 80-90% confluent. When cells were ready the plates were washed in ice-cold PBS and lysed in ice-cold lysis buffer for 5 minutes. Cells were scraped from culture dishes and centrifuged at 13,000 rpm for 6min at 4ºC. The amount of protein present in the supernatant was determined using the bicinchoninic acid assay (BCA). Samples were prepared into aliquots and stored at -80 ºC.

The SOP used for the preparation of the protein lysates used in Western blots is described in Appendix 12. The protein lysates used in this study had been prepared previously and stored at -80°C.

The SOP used to determine the protein concentration in each lysate sample is detailed in Appendix 13. These samples were already prepared and thawed for use when required.

Western Blots

Western blots are used to detect protein levels by separating proteins on a polyacrylamide gel. Antibodies are used to detect the protein and the results are visualised using chemiluminescence.

Aliquots of protein samples were prepared as described previously with 50µg of protein in each sample. A 10% resolving gel was prepared, poured and left to set. 3.6% stacking gel was prepared and poured on top of the resolving gel. A comb was immediately inserted to create wells, the comb removed once gel had set. Protein samples and rainbow and New England markers were denatured by heating for 5 minutes at 95°C and 26µl of each loaded onto the wells. This was run at 120mA for 30 minutes then 70mA for 4 hours. The proteins were transferred to a permeabilised membrane by running at 30v at 4°C overnight. Membranes were blocked by incubating for 1 hour at room temperature using blocking agent diluted 50:50 with PBS. Primary antibody was diluted 1:1000 and incubated overnight at 4°C. From this point all steps were completed in the dark. Membranes were washed for 5 minutes X3 with PBS/tween then incubated for 45 minutes at room temperature with blocking buffer (as described above) and secondary antibody diluted 1:10. Membranes were washed for 5 minutes X3 in PBS/tween and 5 minutes X3 in PBS then blotted and left to dry in complete darkness.

The SOPs used to perform Western blot are detailed in Appendix 14.

The cell samples and ladder markers used for each antibody are shown in Table 2.5.

Cell samples/ ladder markers	Protein volume	Primary Antibody	Secondary Antibody
1) Rainbow marker (161-0324 Biorad) 2) NEB marker (P7708 New England biomarkers) 3) MCF10A 4) BT474 5) MDA MB453 6) MDA MB466 7) SKBR3	1) 26µl 2) 26µl 3-7) 26µl of each sample was loaded with 50µg of protein in each sample	Thymidylate Synth(et)ase (TS) Supplier: Abcam Rabbit mAb, ab29538 Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10
1) Rainbow marker (161-0324 Biorad) 2) NEB marker (P7708 New England biomarkers) 3) MCF10A 4) BT474 5) MDA MB453 6) MDA MB466 7) SKBR3	1) 26µl 2) 26µl 3-7) 26µl of each sample was loaded with 50µg of protein in each sample	Cytidine deaminase (CDA) Supplier: Novus Biologicals Rabbit polyclonal, NBP1-31412 Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10
1) Rainbow marker (161-0324 Biorad) 2) NEB marker (P7708 New England biomarkers) 3) MCF10A 4) BT474 5) MDA MB453 6) MDA MB466 7) SKBR3	1) 26µl 2) 26µl 3-7) 26µl of each sample was loaded with 50µg of protein in each sample	Human equilibrative nucleoside transporter (hENT1) Supplier: Novus Biologicals Rabbit polyclonal Ab, NBP1-50508 Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10

Cell samples/ ladder markers	Protein volume	Primary Antibody	Secondary Antibody
1) Rainbow marker (161-0324 Biorad) 2) NEB marker (P7708 New England biomarkers) 3) MCF10A 4) BT474 5) MDA MB453 6) MDA MB466 7) SKBR3	1) 26µl 2) 26µl 3-7) 26µl of each sample was loaded with 50µg of protein in each sample	Deoxycytidine kinase (DCK) Supplier: Lifespan Biosciences Rabbit polyclonal, LS-B1825 Rabbit polyclonal Ab Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10
1) NEB marker (P7708 New England biomarkers) 2) BT549 3) HBL100 4) HCC1954 5) MCF7 6) MDA MB361 7) MDA MB463 8) T47D 9) ZR75/1 10) Rainbow marker (161-0324 Biorad)	1) 26µl 2-9) 26µl of each sample was loaded with 50µg of protein in each sample 10) 26µl	Ribonucleotide reductase subunit M1 (RRM1) Supplier: Proteintech, Rabbit polyclonal Ab, 10526-1-AP Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10
1) NEB marker (P7708 New England biomarkers) 2) BT549 3) HBL100 4) HCC1954 5) MCF7 6) MDA MB361 7) MDA MB463 8) T47D 9) ZR75/1 10) Rainbow marker (161-0324 Biorad)	1) 26µl 2-9) 26µl of each sample was loaded with 50µg of protein in each sample 10) 26µl	Ribonucleotide reductase subunit M2 (RRM2) Supplier: Abcam, mouse monoclonal AB, ab115701 Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10

Cell samples/ ladder markers	Protein volume	Primary Antibody	Secondary Antibody
1) NEB marker (P7708 New England biomarkers) 2) BT549 3) HBL100 4) HCC1954 5) MDA MB361 6) MDA MB463 7) MDA MB453 8) MDA MB468 9) SKBR3 10) ZR75/1 11) Rainbow marker (161-0324 Biorad)	1) 26µl 2-10) 26µl of each sample was loaded with 50µg of protein in each sample 11) 26µl	Dihydropyrimidine dehydrogenase (DPYD) Supplier: Cell Signaling, Rabbit mAb, #4654 Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10
1) NEB marker (P7708 New England biomarkers) 2) BT549 3) HBL100 4) HCC1954 5) MDA MB361 6) MDA MB463 7) MDA MB453 8) MDA MB468 9) SKBR3 10) ZR75/1 11) Rainbow marker (161-0324 Biorad)	1) 26µl 2-10) 26µl of each sample was loaded with 50µg of protein in each sample 11) 26µl	Thymidine phosphorylase (TP) Supplier: Cell Signaling, Rabbit mAb, #4307 Dilution 1:1000	Goat anti-rabbit fluorescent dye Dilution 1:10

Table 2.7 Cell samples and ladder markers used for each primary antibody

2.3.2.4 Immunohistochemical evaluation of antibody

Sections were dewaxed in 3 changes of xylene for 5 minutes each. Slides were then rehydrated through a series of alcohols – 100%, 100%, 80% and 50% - 2 minutes in each, then washed in running water for 2 minutes.

The 0.1M sodium citrate/0.1M citric acid pH6 method of antigen retrieval was used by microwaving slides in a pressure cooker for 5 minutes on the high setting. These were left to cool then washed in 0.05% PBS/tween for 5 minutes. Endogenous peroxidase was blocked by incubating for 10 minutes with 3% hydrogen peroxide (H_2O_2). Slides were rinsed X2 in 0.05% PBS/tween for 5 minutes and background staining blocked by incubating with Dako total protein blocking solution used undiluted for 10 minutes.

The primary antibody was diluted with Dako antibody diluent at the predetermined optimal dilution and incubation time (see table 2.6). Slides were rinsed X3 in 0.05% PBS/tween for 5 minutes and incubated with Dako envision labelled polymer for 30 minutes. Slides were rinsed X3 in 0.05% PBS/tween for 5 minutes then incubated with diaminobenzidine (DAB) diluted in substrate buffer 1:50 for 10 minutes.

Slides were washed for 10 minutes in running water then stained in Harris haematoxylin for 1 minute and washed in running water for 2 minutes. Slides were then placed in Scott's tap water substitute until the sections turned blue. The nuclei were checked microscopically to ensure the correct level of staining. If too dark the slides were differentiated in acid/alcohol, washed in water and blued in Scott's tap water substitute. If too light they were placed in haematoxylin for a further 10 seconds and washed and blued as previously described. Once the correct level of nuclear staining was achieved the slides were washed in running water for 2 minutes.

The slide rack was allowed to drain well and slides dehydrated through alcohols – 50%, 80%, 100% and 100% - for 30 seconds, 30 seconds, 2 minutes, 2 minutes.

Slides were cleared in 3 changes of xylene, 5 minutes each and mounted using DPX.

Each antibody was stained using the manufacturers' recommended positive controls wherever possible and a negative control was also included in every antibody staining run. The method used for IHC can be found in Appendix 15.

The sections were analysed to check that:

a) The staining was "clean", there was no background non-specific staining.

b) The target area of the cell was stained by the antibody.

Steps A and B were double-checked by a Biomedical Scientist to ensure there was no researcher bias.

See Appendix 16 for an example of the dilutions used and summary of steps for antibody optimisation. See table 2.6 below for the final antibody optimal conditions for use.

Antibody name	Supplier	Cat No.	Species	Antigen retrieval	IHC dilution	Incubation time and condition
hENT1	Abcam	ab135756	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:50	1 hour at RT
CDA	Abcam	ab82347	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:200	1 hour at RT
DCK	Abcam	ab151996	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:25	Overnight at 4^{o}C
TS	Abcam	ab108995	Rabbit monoclonal	Sodium citrate/ citric acid pH6	1:25	1 hour at RT
RRM1	Protein Tech	10526-1-AP	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:100	1 hour at RT
RRM2	Novus Biologicals	NBP1-31661	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:100	1 hour at RT
DPYD	Cell Signaling	4654s	Rabbit monoclonal	Sodium citrate/ citric acid pH6	1:50	1 hour at RT
TP	Cell Signaling	4307s	Rabbit monoclonal	Sodium citrate/ citric acid pH6	1:25	1 hour at RT

Table 2.8 Primary antibody optimal conditions for use in IHC

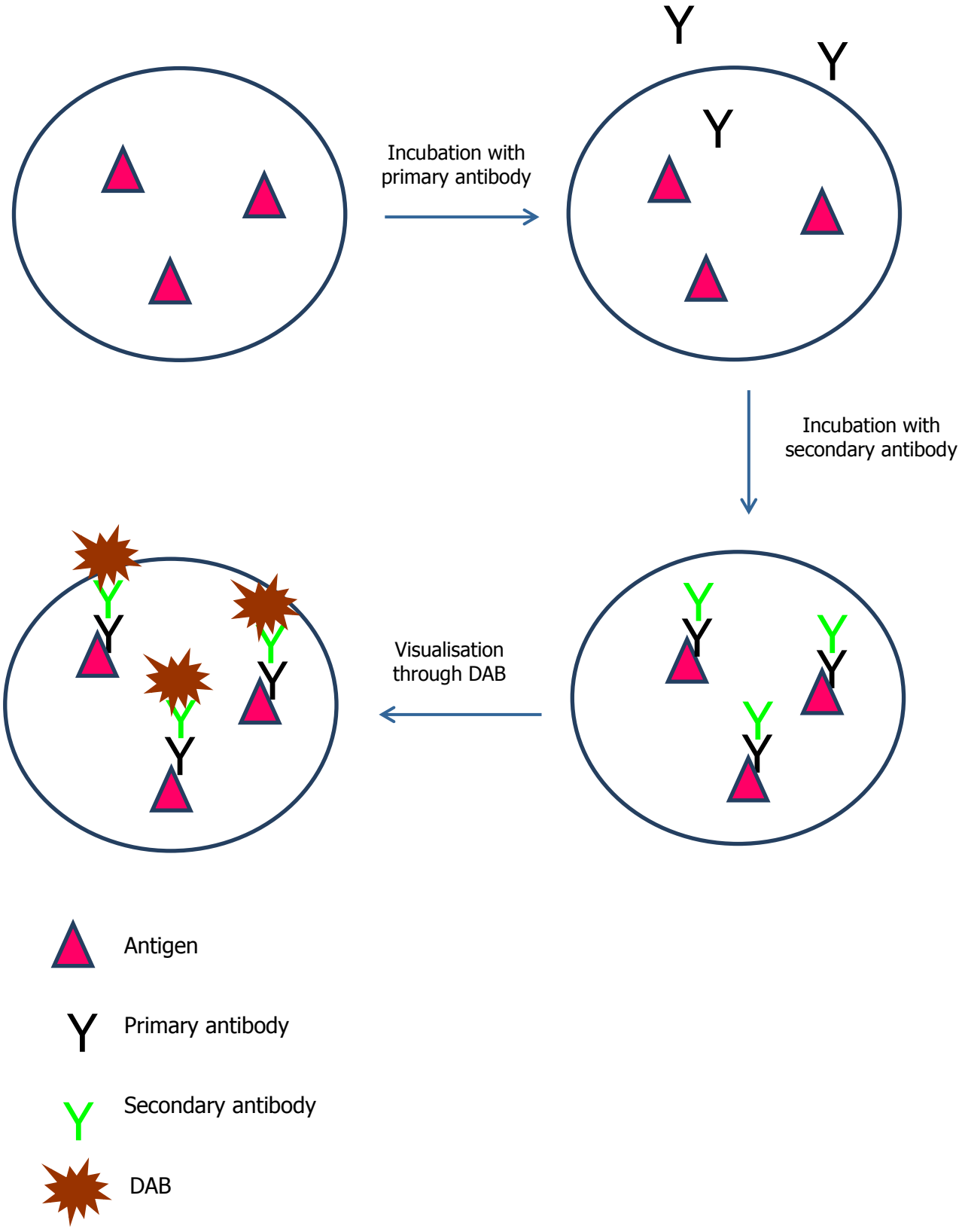
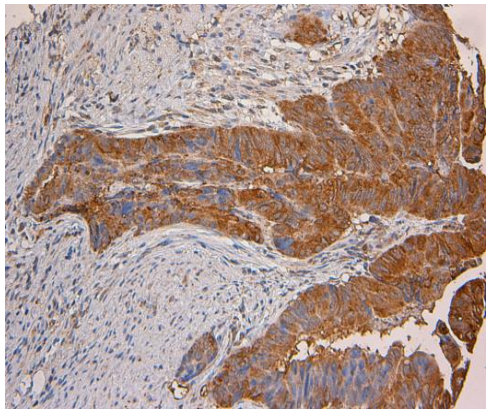
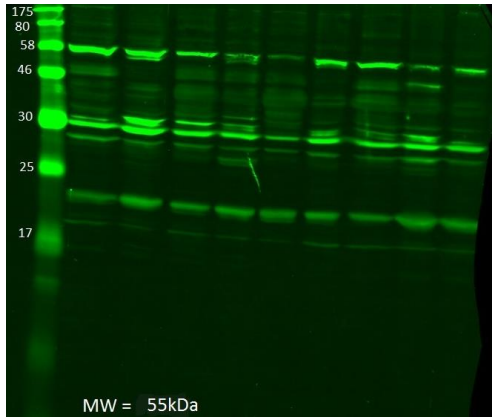
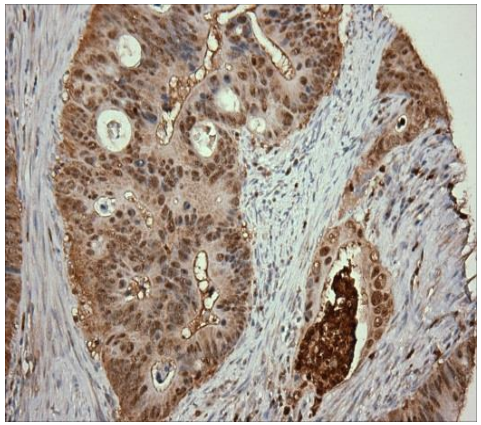


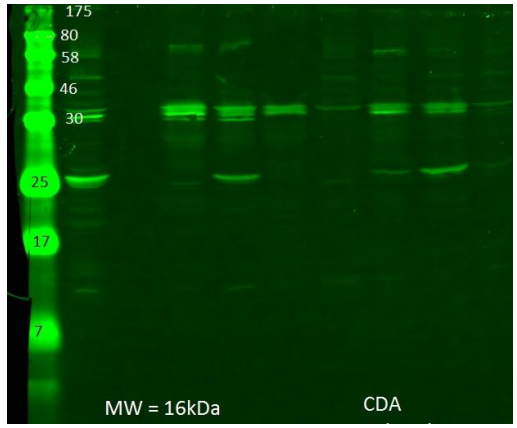
Figure 2.4 Simplified IHC process to show how protein is visualised



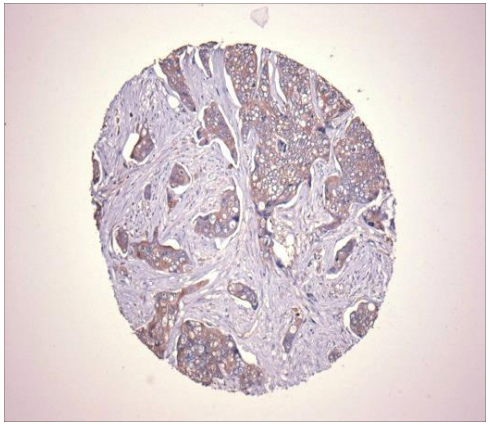


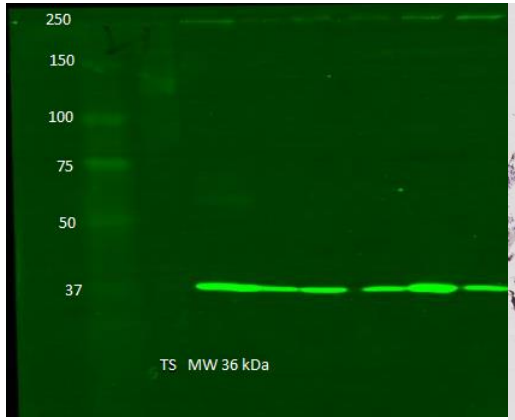
hENT1 Epitomics Cat # T0108. Predicted MW 55kDa



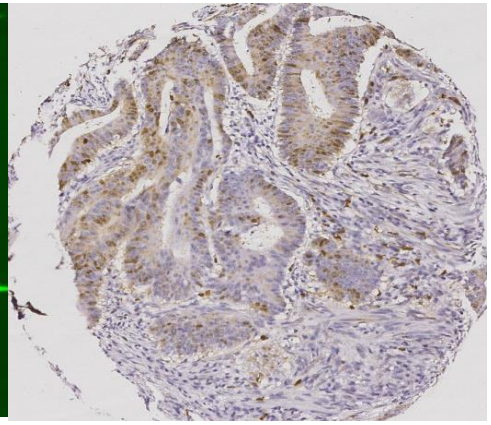


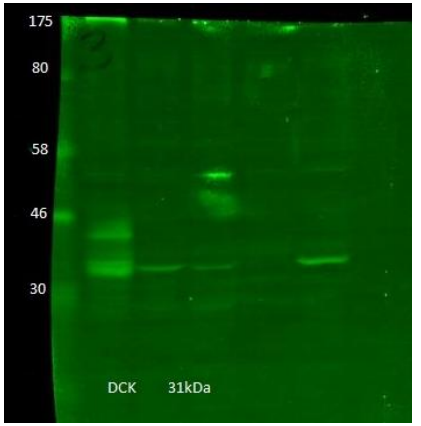
CDA Epitomics Cat # T1049. Predicted MW 16kDa



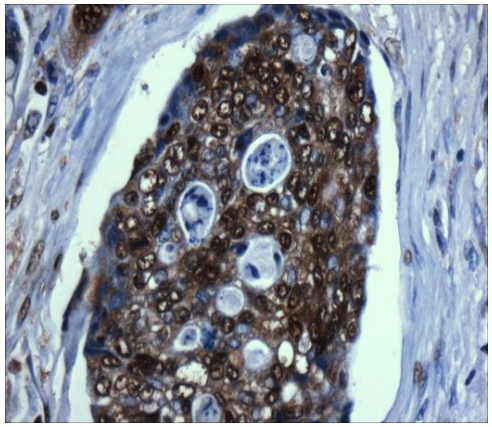


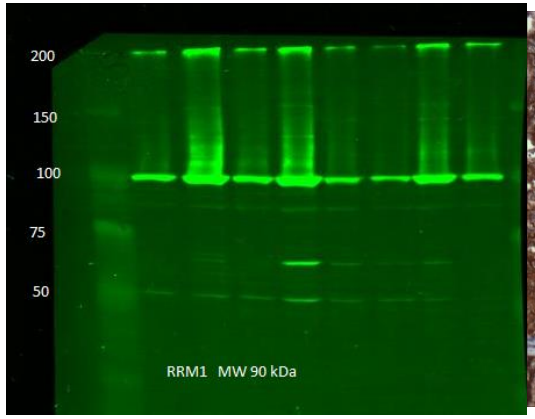
TS Abcam Cat # ab108995. Predicted MW 36kDa



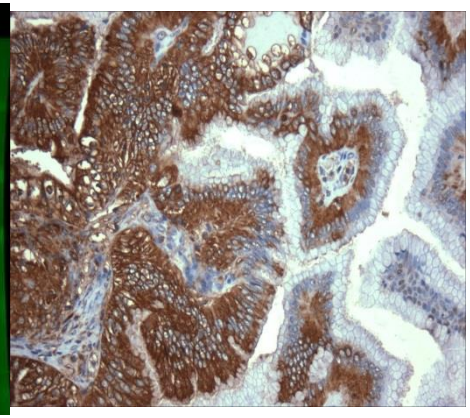


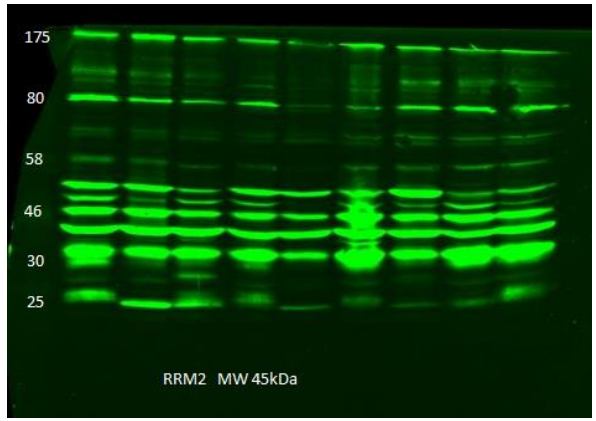
DCK Lifespan Bioscience Cat # LS-B1825. Predicted MW 31kDa



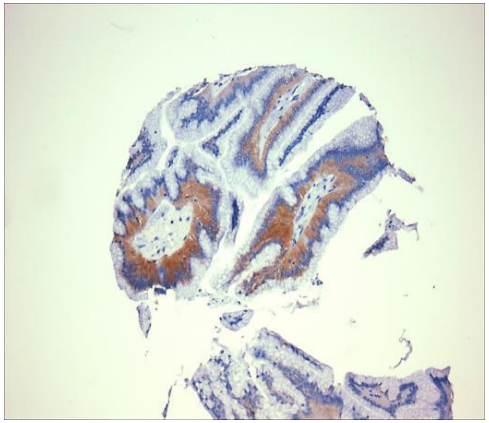


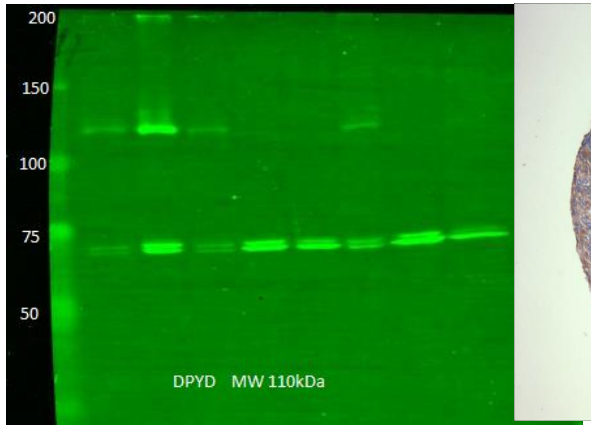
RRM1 ProteinTech Cat # 10526-1-AP. Predicted MW 90kDa



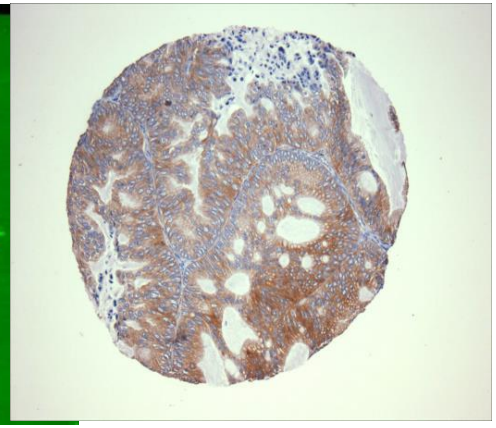


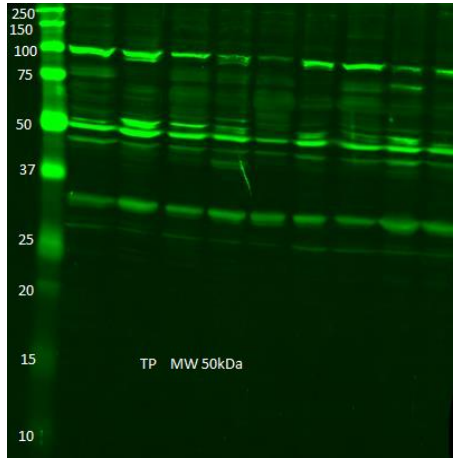
RRM2 Abcam Cat # ab115701. Predicted MW 45 kDa





DPYD Cell Signaling Cat # 4654s. Predicted MW 110kDa





TP Cell Signaling Cat # 4307s. Predicted MW 50kDa

Figure 2.5 IHC and Western blot used as part of antibody validation

The figure above shows the Western blots and IHC performed as part of the antibody validation process. The final lab work was undertaken at St. Andrews medical school labs using antibodies that had been validated there by lab personnel. The table below shows the final antibodies used.

ANTIBODY	SUPPLIER	Cat#	Antibodypedia score	Western blot score	IHC target score	Vendor validation score	TOTAL (30 points is a PASS)	RESULT
hENT1	Abcam	ab135756	5	10	10	5	30	PASS
CDA	Abcam	ab82347	5	10	10	5	30	PASS
DCK	Abcam	ab151996	5	10	10	5	30	PASS
TS	Abcam	ab108995	10	10	10	5	35	PASS
RRM1	Protein Tech	10526-1-AP	10	10	10	10	40	PASS
RRM2	Novus Biologicals	NBP1-31661	10	10	10	10	40	PASS
DPYD	Cell Signaling	4654s	10	10	10	5	35	PASS
TP	Cell Signaling	4307s	10	10	10	5	35	PASS

Table 2.9 The final selection of antibodies used and the level of validation achieved.

These antibodies were used to stain the sets of four TMAs using an immunofluorescence technique.

2.3.3 Immunofluorescence

Sections were dewaxed in 3 changes of xylene for 5 minutes each. Slides were then rehydrated through a series of alcohols – 100%, 100%, 80% and 50% - 2 minutes in each, then washed in running water for 2 minutes.

The 0.1M sodium citrate/0.1M citric acid pH6 method of antigen retrieval was used by microwaving slides in a pressure cooker for 5 minutes on the high setting. These were left to cool then washed X2 in 0.05% PBS/tween for 5 minutes. Endogenous peroxidase was blocked by incubating for 10 minutes with 3% hydrogen peroxide (H2O2). Slides were rinsed X2 in 0.05% PBS/tween for 5 minutes and background staining blocked by incubating with Dako total protein blocking solution used undiluted for 10 minutes. The primary antibody was diluted as previously detailed and the mouse anti-cytokeratin tumour mask was diluted 1:50 with Dako antibody diluent and incubated overnight at 4°C. Slides were rinsed X3 in 0.05% PBS/tween for 5 minutes and incubated for 1.5 hours in the dark with goat anti-mouse Alexa 555 diluted 1:25 in goat-rabbit HRP antibody solution. All steps from hereon in were performed on the dark. Slides were rinsed X3 in 0.05% PBS/tween for 5 minutes then treated with Cy5 tyramide diluted 1:50 in target signal amplification diluent for 10 minutes. Slides were rinsed X3 in 0.05% PBS/tween for 5 minutes then placed in 80% ethanol for 1 minute. Slides were left to air dry in complete darkness overnight. Prolong gold anti-fade reagent with DAPI was used as a mounting agent and the slides coverslipped and left to dry overnight in complete darkness.

See Appendix 17 for the method used to stain the sections using immunofluorescence (IF). Each antibody was optimised (as previously described for IHC) and the optimal dilution decided prior to staining TMAs.

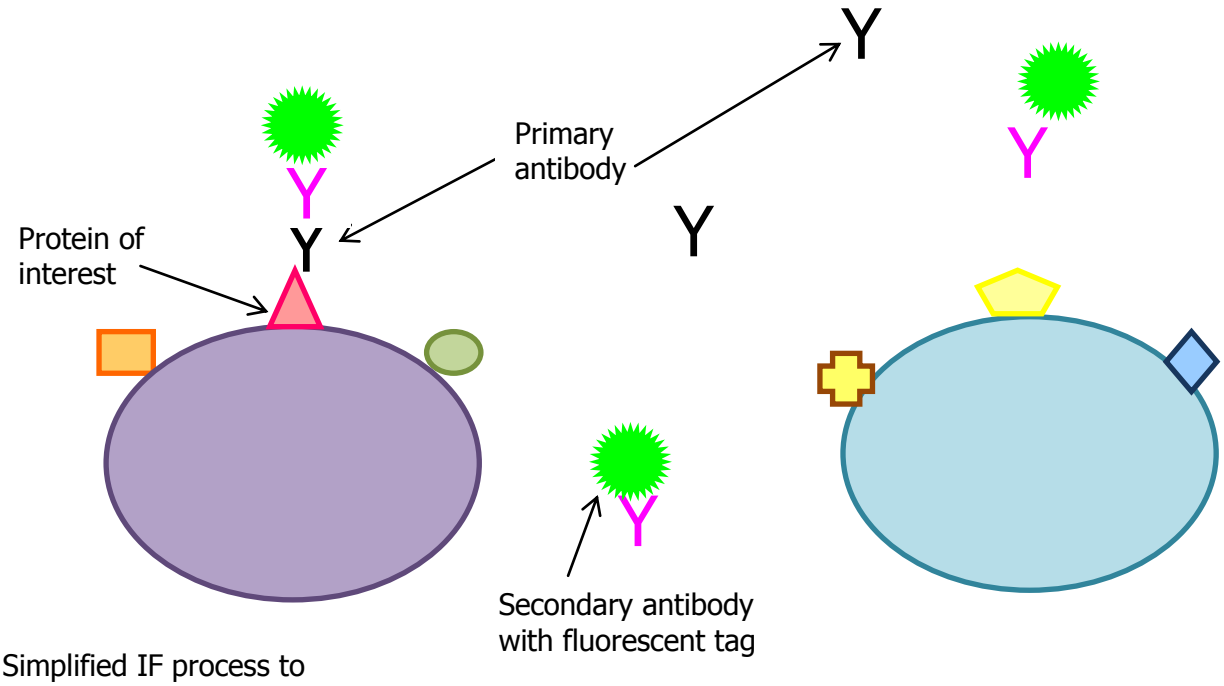


Figure 2.6 Simplified IF process to show how protein is visualised

Antibody name	Supplier	Cat No.	Species	Antigen retrieval	IF dilution	Incubation time and condition
hENT1	Abcam	ab135756	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:500	Overnight at 4°C
CDA	Abcam	ab82347	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:250	Overnight at 4°C
DCK	Abcam	ab151996	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:750	Overnight at 4°C
TS	Abcam	ab108995	Rabbit monoclonal	Sodium citrate/ citric acid pH6	1:100	Overnight at 4°C
RRM1	Protein Tech	10526-1-AP	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:250	Overnight at 4°C
RRM2	Novus Biologicals	NBP1-31661	Rabbit polyclonal	Sodium citrate/ citric acid pH6	1:500	Overnight at 4°C
DPYD	Cell Signaling	4654s	Rabbit monoclonal	Sodium citrate/ citric acid pH6	1:50	Overnight at 4°C
TP	Cell Signaling	4307s	Rabbit monoclonal	Sodium citrate/ citric acid pH6	1:500	Overnight at 4°C

Table 2.10 Finalised list of primary antibody used at St. Andrews for IF

2.3.4 AQUA Automated QUantitative Analysis™

Figure 2.7 AQUA image analysis hardware

Because of the subjectivity of IHC scoring there are a number of computer-based quantitative analysis packages commercially available.

The AQUA system was first developed in the Department of Pathology at Yale University. [125] AQUA utilises an immunofluorescent (IF) based technique rather than the conventional chromogenic staining to visualise protein expression. Firstly, a cytokeratin mask is applied to the section to enable differentiation between tumour cells and cell stroma. Then antibody-conjugated fluoresecent dye is used to stain the target protein, instead of the diaminobenzidine (DAB)/immunoperoxidase (or brown staining) used in conventional IHC. Finally, a nuclear counterstain is used, which is 4,6-Diamidino-2-phenylindole (DAPI) for AQUA instead of haematoxylin which is used in conventional IHC.

Camp et al devised a series of algorithms that he called AQUA which allow rapid, high-throughput analysis of TMAs.

The first algorithm, called PLACE (Pixel based Locale Assignment for Compartmentalisation of Expression), separates tumour cells from the stroma and defines the subcellular compartments using fluorescent labels. The target protein is then quantified and located using these fluorescent tags. The amount of protein expressed is calculated by dividing the intensity of fluorescence by the total area of tumour tissue scanned.

The second algorithm, called RESA (Rapid Exponential Subtraction Algorithm), allows for analysis of overlapping subcellular compartments, despite the thickness of the tissue

sections, by subtracting an out-of-focus image from a focussed one based on pixel intensity, signal-to-noise ratio, and the expected compartment size. [125]

These algorithms were validated using oestrogen receptor positive (ER positive) breast cancer tissue and colon carcinoma comparing pathologist scoring with AQUA quantification. The theory behind AQUA is to visualise and quantify the protein of interest within each tumour core. Cytokeratin (Cy3) is used to differentiate the epithelial tumour cells from the stroma, commonly referred to as the "tumour mask". It also allows subcellular localisation of the target protein in combination with DAPI, which is used to stain the nuclei. [112] That is, any area within the tumour mask area not stained with DAPI is deemed cytoplasmic.

Cy5 is the fluorophore of choice for the target protein as it is brighter than other fluorescein dyes and there is minimal auto-fluorescence of tissue at this wavelength (670nm). The reason for this is because intrinsic and induced auto-fluorescence emits at a wavelength of 450-650nm and therefore does not compete with Cy5. [126]

AQUA not only quantifies the target protein expression within the tumour area, it differentiates between the nuclear and cytoplasmic expression. Any cores lacking sufficient tumour (<5% of total area has cytokeratin mask) are automatically excluded.

See Appendices 18 and 19 to see the methods used for AQUA image acquisition and AQUA image analysis respectively.

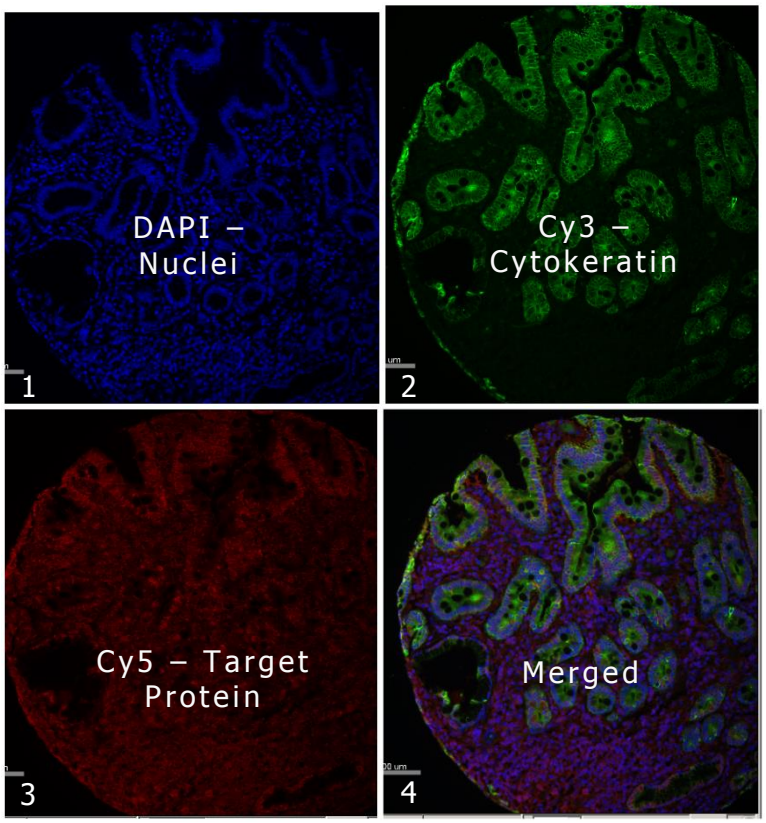


Figure 2.8 TMA core stained for AQUA

Picture 1 shows nuclei only stained with DAPI

Picture 2 shows the cytokeratin tumour mask – cytokeratin is used to determine the areas of each TMA core that are tumour

Picture 3 shows the target protein expression in the same core

Picture 4 shows pictures 1-3 merged so we can see target protein expression in the tumour area as well as the stroma

2.3.5 Statistical analysis

The statistical packages TMA Navigator and SPSS v21 were used for univariate and multivariate analysis.

2.3.5.1 Lecca Formula

Ozan Kahramanogullari, Gianluca Fantaccini, Paola Lecca, Daniele Morpurgo and Corrado Priami created an algorithmic model to quantify the metabolic inhibitions to gemcitabine efficacy.[128] The nucleoside transporter hENT1 was deemed an independent factor in gemcitabine resistance as it is responsible for the transport of gemcitabine into the cell. DCK is responsible for the phosphorylation of gemcitabine to gemcitabine monophosphate. RRM1 and RRM2 are responsible for catalysing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides. Thus it is reasonable to hypothesise that increased survival time could be associated with high DCK and low RRM1/ RRM2 expression.

Lecca et al created a final equation using DCK, RRM1 and RRM2:

DCK/(RRM1*RRM2)

This equation was used as a means of multivariate analysis.

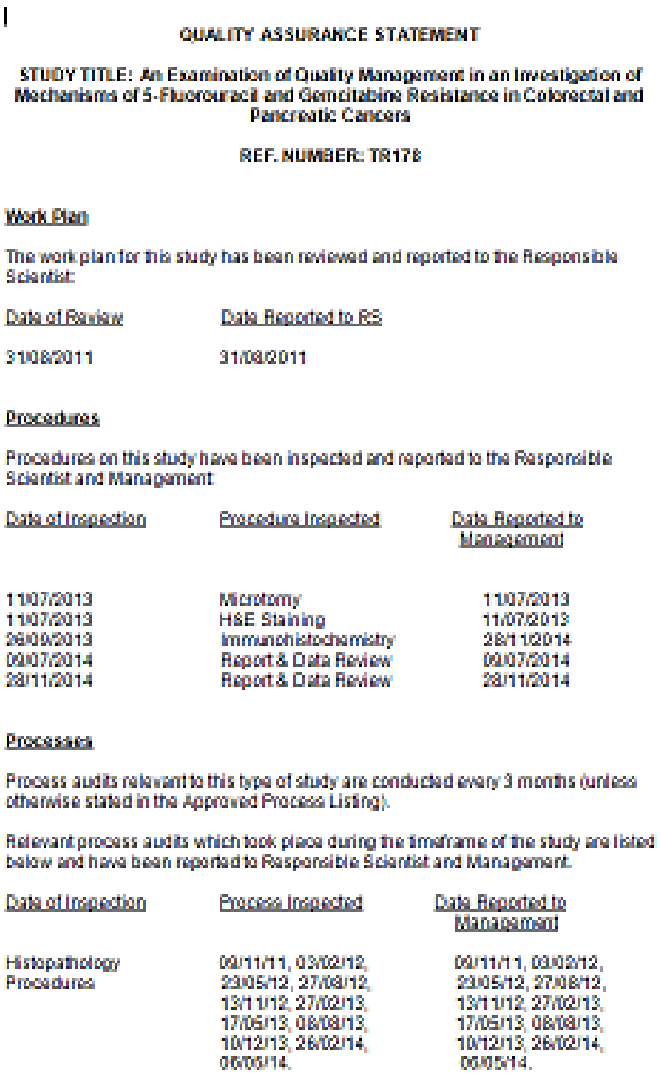
3. Results

3.1 Quality results

3.1.1 Quality Assurance statement

A QA programme was maintained throughout the duration of this project which included process inspections (these audit lab work undertaken against the relevant SOP) and data checking (to ensure accuracy and integrity of data) and final report checking (to ensure that the results generated were valid).

A QA statement was written at the end of the study to confirm the integrity of the work undertaken and the data generated. The full QA statement can be seen in Appendix 20.



QUALITY ASSURANCE STATEMENT

STUDY TITLE: An Examination of Quality Management in an Investigation of Mechanisms of 5-Fluorouracil and Gemcitabine Resistance in Colorectal and Pancreatic Cancers

REF. NUMBER: TR178

Work Plan

The work plan for this study has been reviewed and reported to the Responsible Scientist:

Date of Review	Date Reported to RS
31/08/2011	31/08/2011

Procedures

Procedures on this study have been inspected and reported to the Responsible Scientist and Management:

Date of Inspection	Procedure Inspected	Date Reported to Management
11/07/2013	Microtomy	11/07/2013
11/07/2013	H&E Staining	11/07/2013
26/09/2013	Immunohistochemistry	28/11/2014
09/07/2014	Report & Data Review	09/07/2014
28/11/2014	Report & Data Review	28/11/2014

Processes

Process audits relevant to this type of study are conducted every 3 months (unless otherwise stated in the Approved Process Listing).

Relevant process audits which took place during the timeframe of the study are listed below and have been reported to Responsible Scientist and Management.

Date of Inspection	Process Inspected	Date Reported to Management
Histopathology Procedures	09/11/11, 03/02/12, 23/05/12, 27/08/12, 13/11/12, 27/02/13, 17/05/13, 08/08/13, 10/12/13, 26/02/14, 06/05/14.	09/11/11, 03/02/12, 23/05/12, 27/08/12, 13/11/12, 27/02/13, 17/05/13, 08/08/13, 10/12/13, 26/02/14, 06/05/14.

Figure 3.1 QA statement

The full QA statement can be found in Appendix 20.

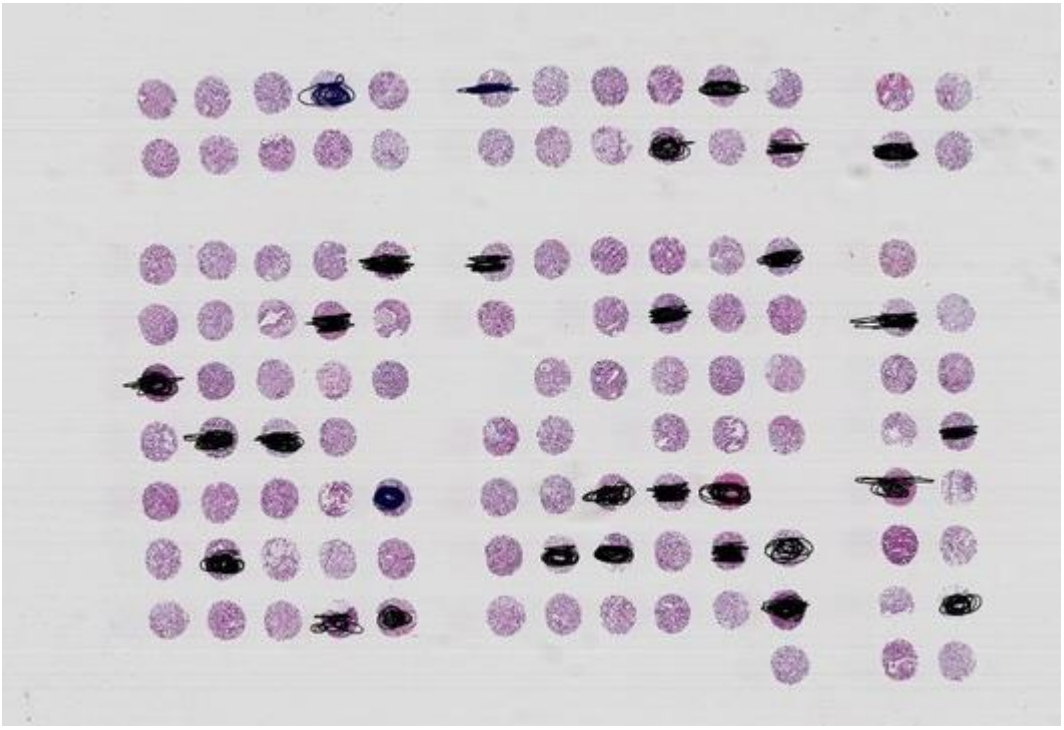


Figure 3.2 Slidepath image of pancreatic cancer TMA cores checked by the pathologist to indicate any cores that were not cancer. These cores were not included in the analysis.

3.1.2 Comparison of AQUA scores between St. Andrews and Edinburgh

The graphs below show the results of pancreatic cancer TMA1 stained using CDA. The slides were scanned using the AQUA facility at both St. Andrews and Edinburgh labs and the expression levels from both plotted. This was undertaken to ensure correlation of results from the two pieces of equipment.

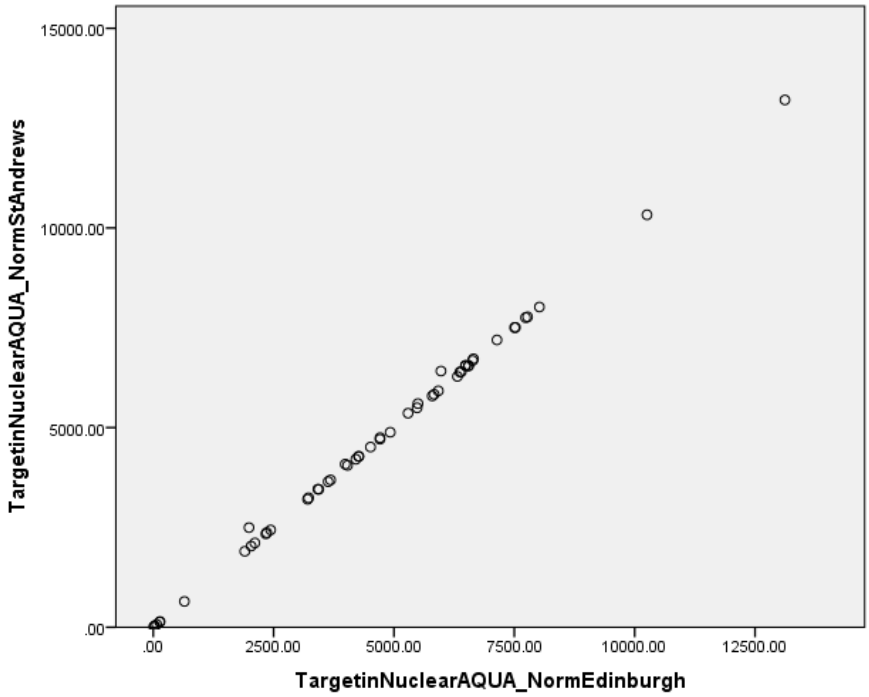


Figure 3.3 Comparison of AQUA nuclear scores between Edinburgh and St. Andrews

The correlation coefficient – or r – of the above scores is 0.999455, which shows a strongly positive correlation. The coefficient of determination – or r^2 – is 0.9989103. This means that 99.89 % of the total variation in Edinburgh scores can be explained by the linear relationship between St Andrews and Edinburgh scores.

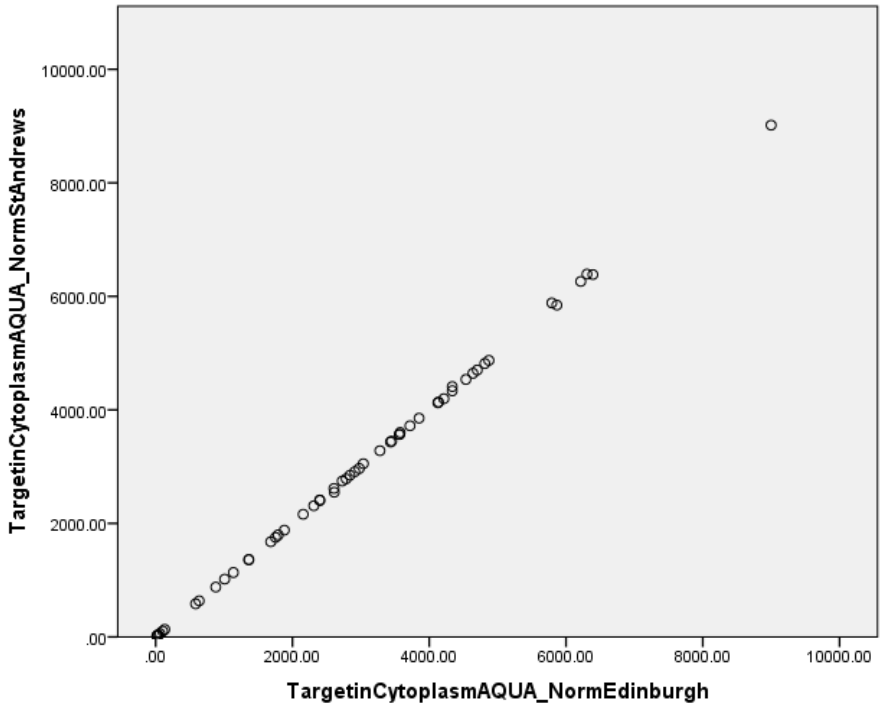


Figure 3.4 Comparison of AQUA cytoplasm scores between Edinburgh and St. Andrews

The correlation coefficient – or r – of the above scores is 0.999940107, which shows a strongly positive correlation. The coefficient of determination – or r2 – is 0.99988022. This means that 99.99% of the total variation in Edinburgh scores can be explained by the linear relationship between St Andrews and Edinburgh scores.

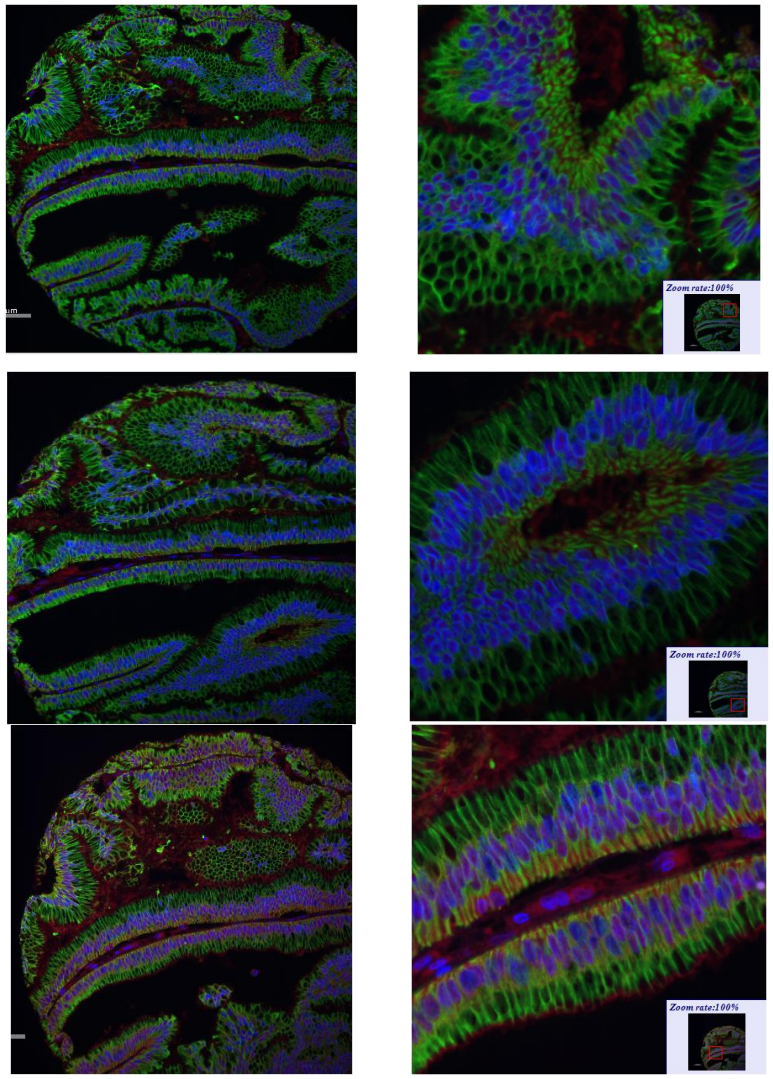
3.1.3 TMA Slide Storage Conditions

The long-term storage of TMA slides may result in a loss of antigenicity, presumably due to oxidation of exposed proteins.

TMA slides were cut, wrapped in foil and stored for 6 months at RT, 4°C and -20°C. The slides were then stained for IF using the method described in Chapter 2 using the TP antibody. The expression levels varied according to the temperature they were stored at, and mostly showed that the lower the temperature the higher the expression levels.

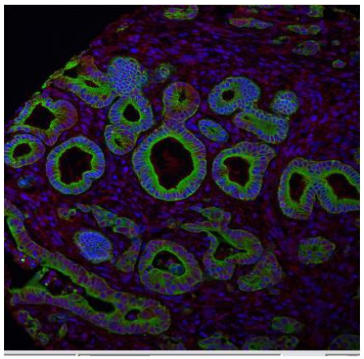
Interpreting these results would indicate that the optimum storage conditions for cut TMA sections are -20^{o}C.

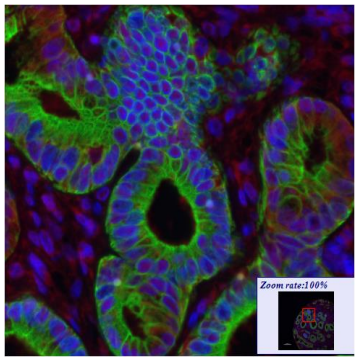
The pictures below show AQUA generated images of sections stored under the 3 different conditions. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.



	AQUA nuclear expression	AQUA cytoplasm expression
Stored at room temperature showing nuclear expression in left hand image and higher magnification in right hand image	6561.7	3877.0
Stored at 4°C showing nuclear expression in left hand image and higher magnification in right hand image	6917.3	4791.5
Stored at -20°C showing nuclear expression in left hand image and higher magnification in right hand image	15312.7	10726.2

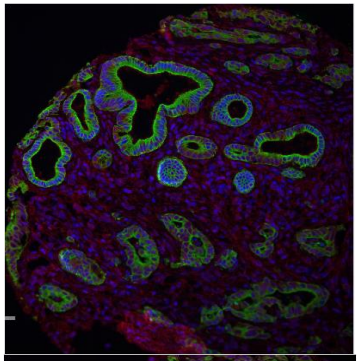
Figure 3.5 Pancreatic cancer TMA1 spot 56 stained with TP after storage for 6 months at RT, 4°C and -20°C. The values for AQUA nuclear and cytoplasm expression are given. Expression levels increase as the temperature decreases, indicating -20°C is the optimum long term storage temperature for preserving antigenicity of cut TMA sections.

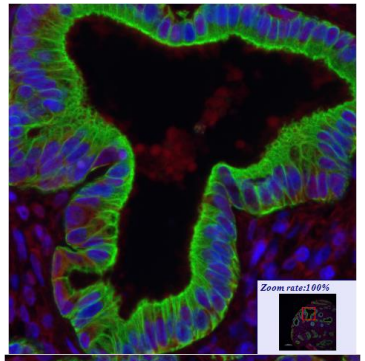




Stored at room temperature showing nuclear expression in left hand image and higher magnification in right hand image

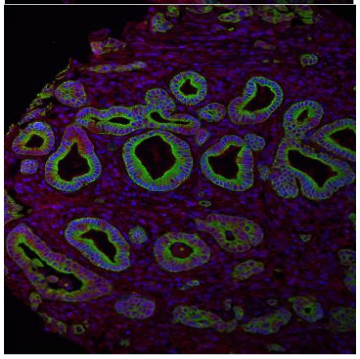
AQUA nuclear expression	AQUA cytoplasm expression
9498.9	7990.7

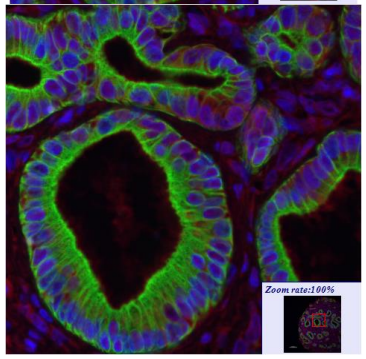




Stored at 4°C showing nuclear expression in left in right hand image and higher magnification in right hand image

10323.9	8700.2

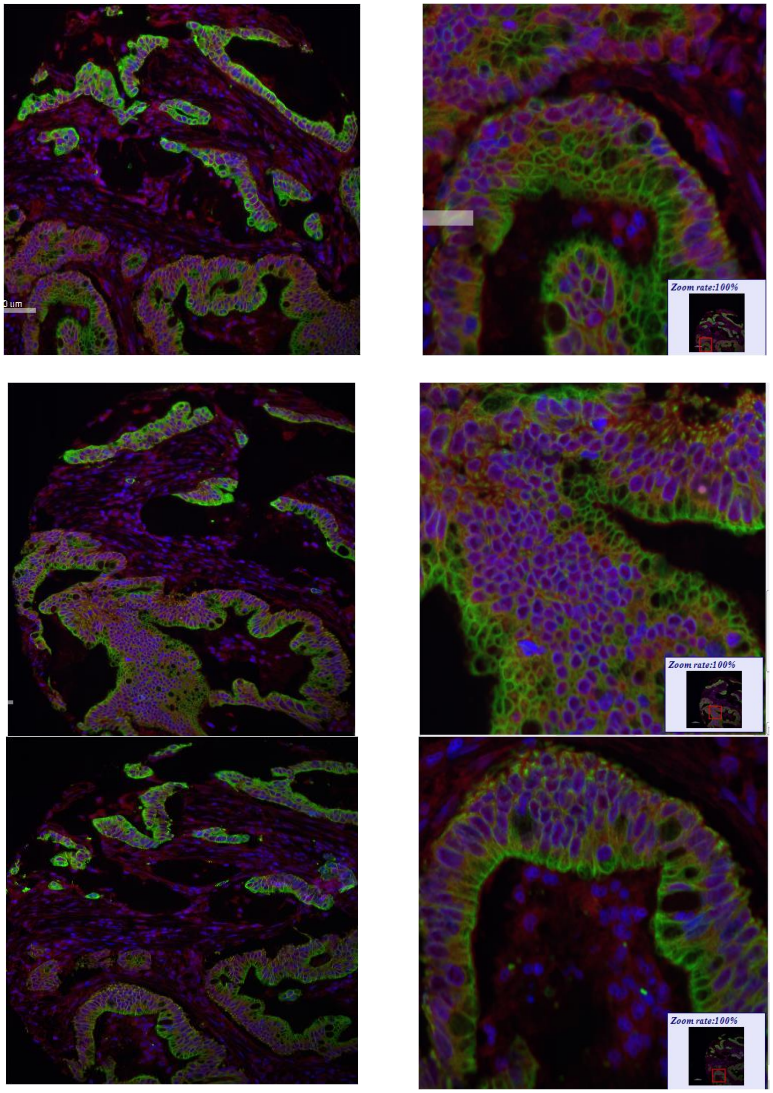




Stored at -20°C showing nuclear expression in left hand image and higher magnification in right hand image

14517.0	12865.9

Figure 3.6 Pancreatic cancer TMA1 spot 62 stained with TP after storage for 6 months at RT, 4°C and -20°C. The values for AQUA nuclear and cytoplasm expression are given. The values for AQUA nuclear and cytoplasm expression are given. Expression levels increase as the temperature decreases, indicating -20°C is the optimum long term storage temperature for preserving antigenicity of cut TMA sections.



	AQUA nuclear expression	AQUA cytoplasm expression
Stored at room temperature showing nuclear expression in left hand image and higher magnification in right hand image	14784.4	11320.6
Stored at 4°C showing nuclear expression in left hand image and higher magnification in right hand image	15389.0	11944.4
Stored at -20°C showing nuclear expression in left hand image and higher magnification in right hand image	13411.4	10513.5

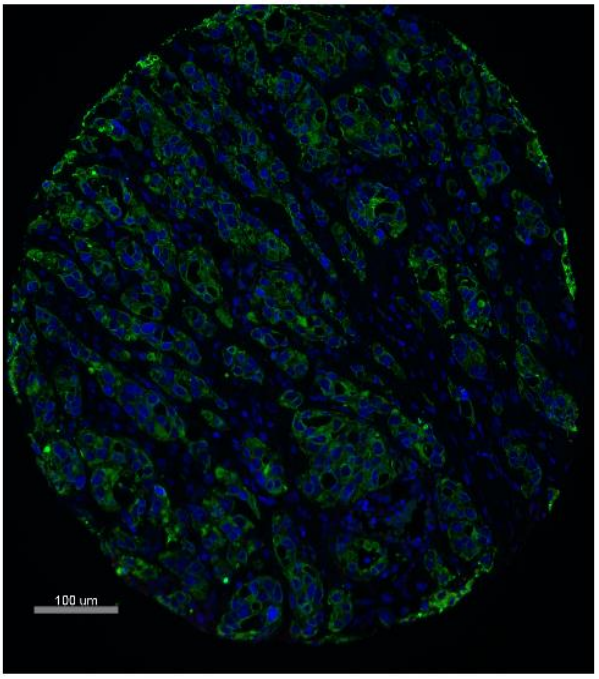
Figure 3.7 Pancreatic cancer TMA1 spot 92 stained with TP after storage for 6 months at RT, 4°C and -20°C. The values for AQUA nuclear and cytoplasm expression are given. The values for AQUA nuclear and cytoplasm expression are given. The cytoplasm expressions are similar at all temperatures with the highest expression level at 4oC. The nuclear expression levels concur, with the highest expression at 4°C. Interestingly, the lowest expression level is at -20°C storage which contradicts the other results.

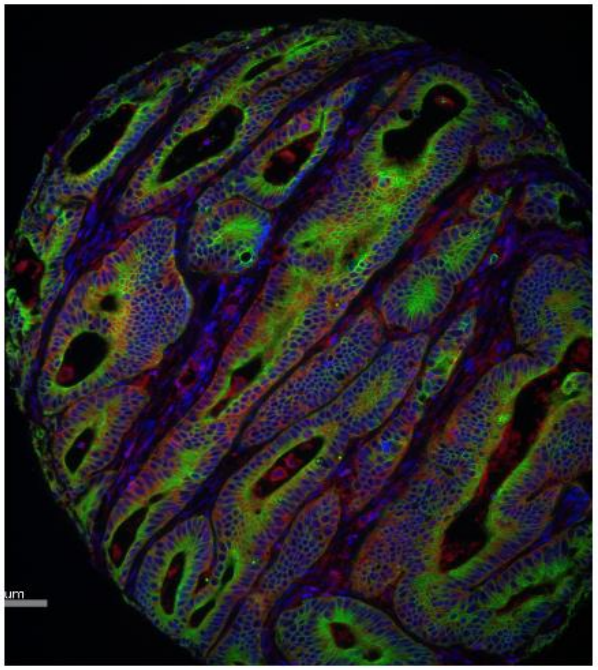
3.2 Pancreatic cancer and gemcitabine resistance results

Variables	Number of patients	Per cent %
Gender	**85**	**100**
Male	46	54
Female	39	46
Age (years)		
Median	66	-
Range	43 - 84	-
Tumour location		
Head/tail/ampulla/common bile duct	65/3/5/2/10	76.5/3.5/6/2.5/11.5
Maximum tumour diameter (mm)		
Mean (+/- SD)	35.56 +/- 10.68	-
Median	32	-
Range	15 - 72	-
Pathologic stage		
T1/T2/T3/T4	3/6/74/2	3.5/7/87/2.5
N0/N1/Nx	15/68/2	17.5/80/2.5
MX/M1/NK	31/2/52	36.5/2.5/61
Tumour differentiation		
Moderate/moderate-poor/poor/well	36/11/28/10	42.5/13/33/11.5
Surgical margins		
Positive/negative	64/21	75/25
Perineural invasion		
Yes/no/NK	70/9/6	82.5/10.5/7
Lymphovascular invasion		
Yes/no/NK	65/14/6	76.5/16.5/7
Pancreatitis		
Yes/no	31/54	36.5/63.5

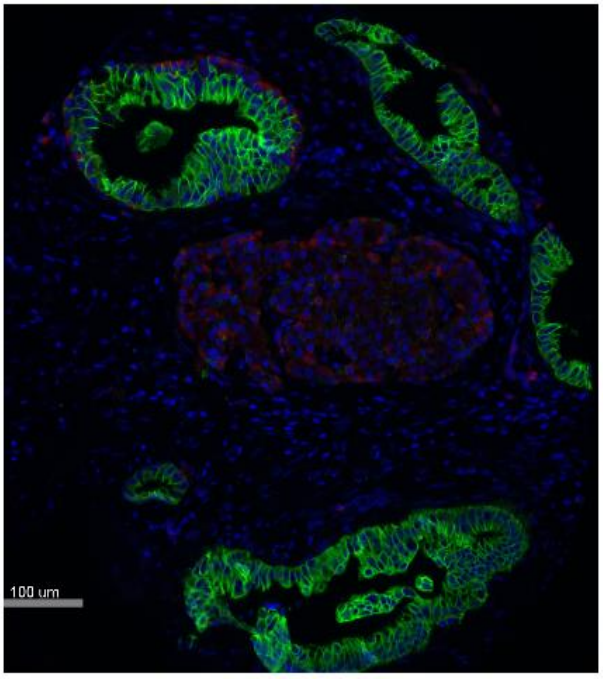
Table 3.1 Patient demographics, clinical characteristics, and histopathological features for pancreatic cancer cohort combat data

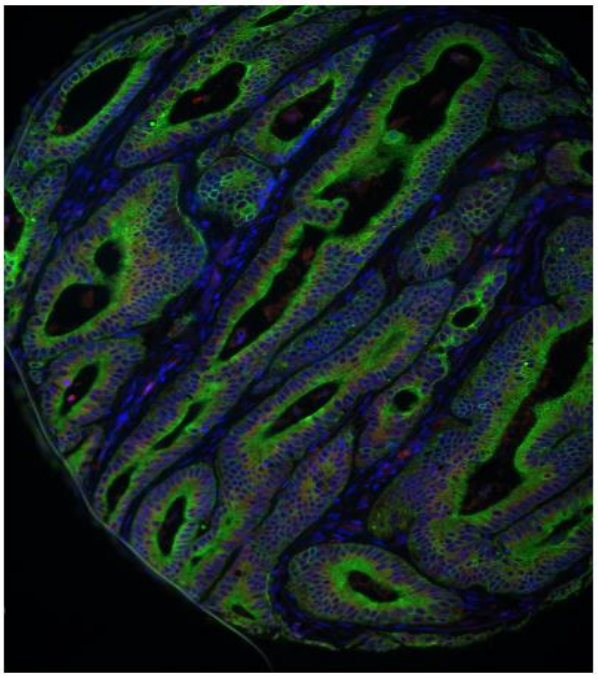
The above demographics were collated by the Tissue Governance team in NHS Lothian. Results are shown as a percentage of the total in the final column.
There is an almost even split between the sexes, with 54% males and 46% females. Ages ranged from 43 – 84 with the median age of 66 and the most common location for tumours being the head of the pancreas (76.5% of the cohort). Most patients were diagnosed at a late stage of the disease – 87% at pathologic stage T3 and 68% with nodal involvement. Due to the proximity of the pancreas to other organs 75% of patients had positive resection margins.
Interestingly, 82.5% of patients showed perineural invasion and 76.5% showed lymphovascular invasion.



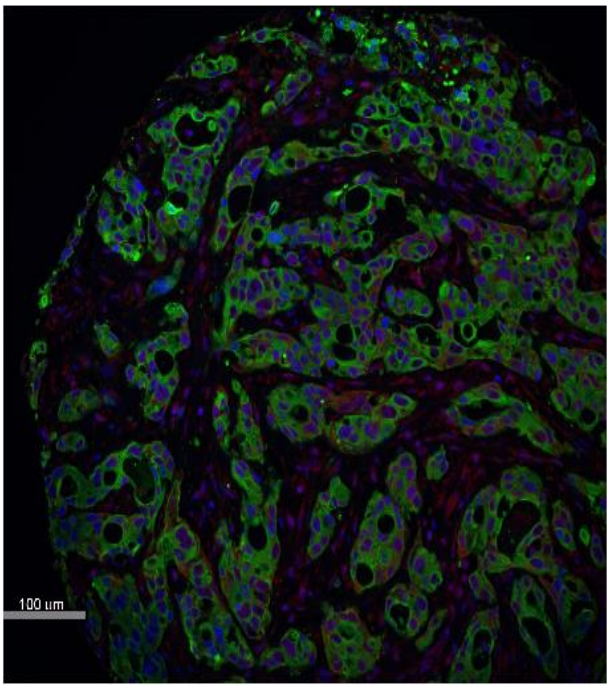


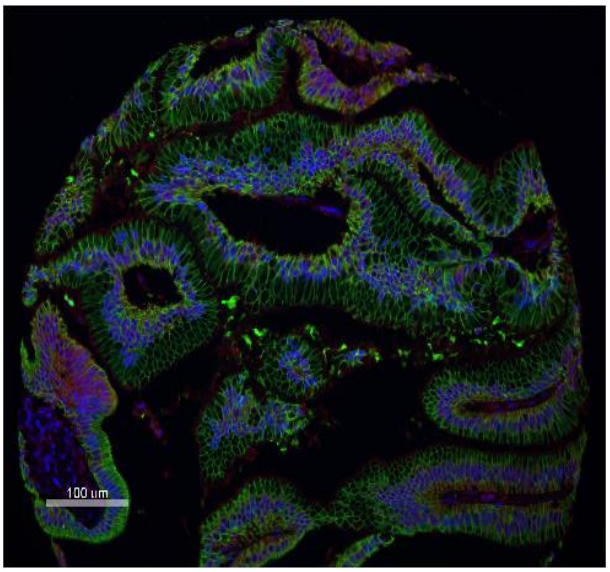
hENT1



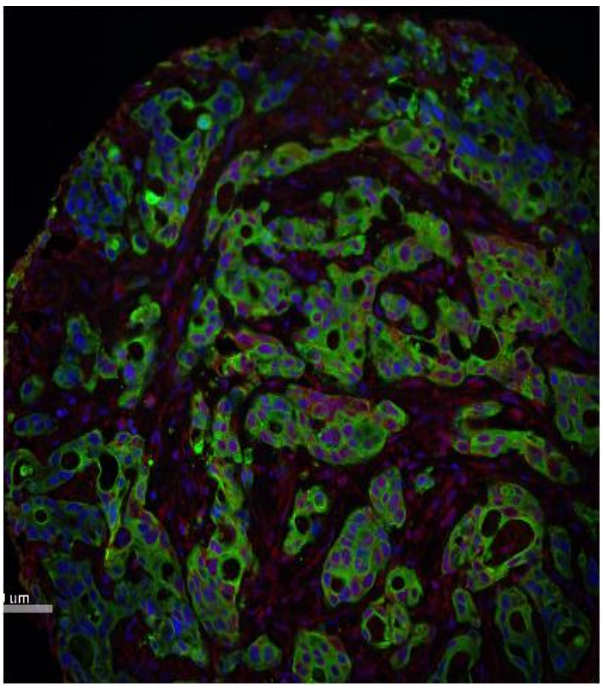


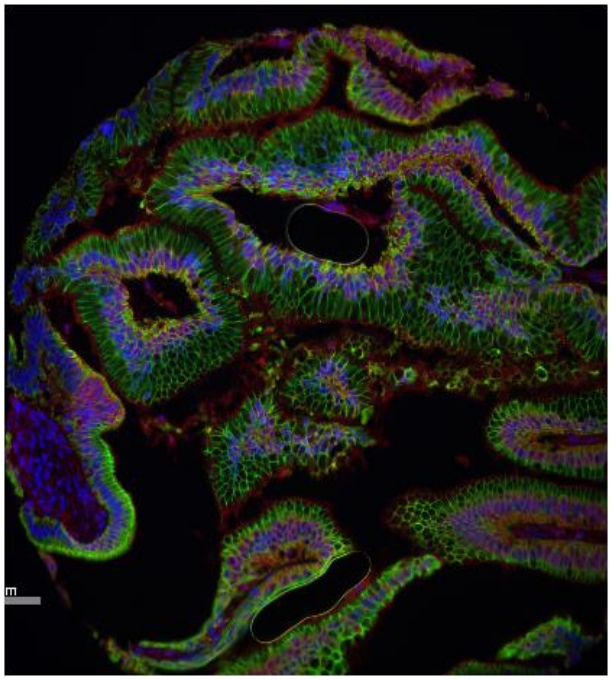
CDA



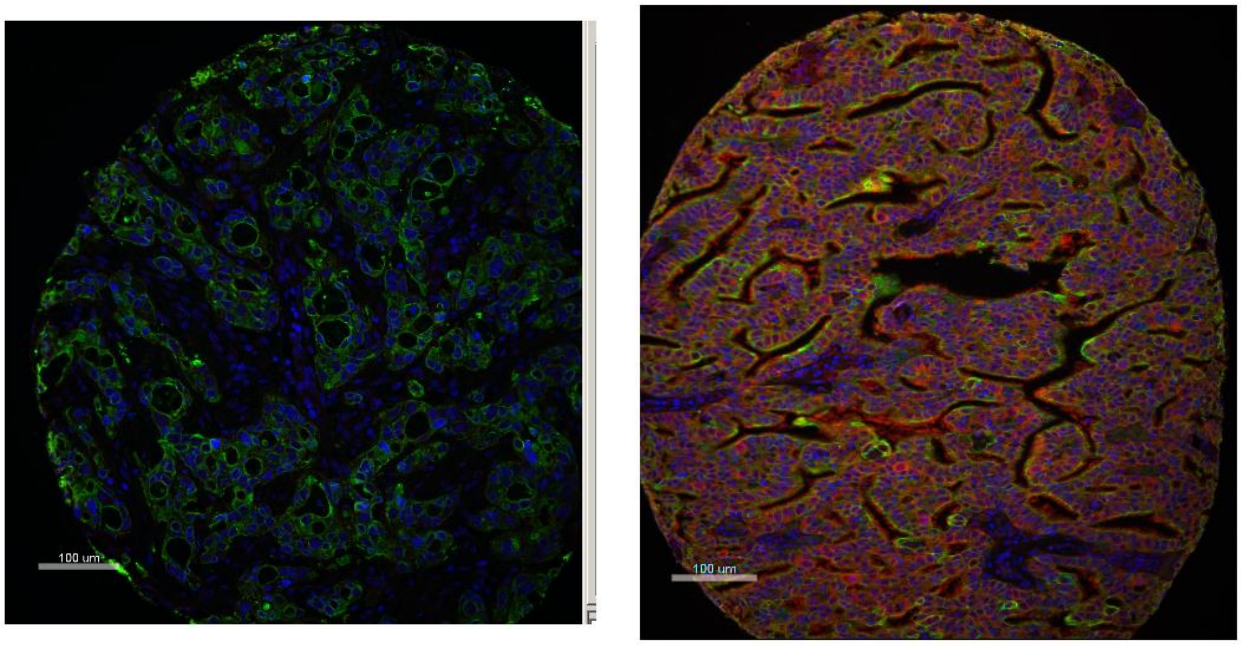


DCK





RRM1



RRM2

Figure 3.8 AQUA immunofluorescence images showing expression of the 5 proteins involved with gemcitabine transport and metabolism

Figure 3.8 above shows the 5 proteins involved in the transport and metabolism of gemcitabine. The cores on the left show low expression and the cores on the right show high expression. These pictures were generated using AQUA software and show the dynamic range of protein expression levels.

3.3 Statistical analysis

3.3.1 Median values versus complete dataset

After consideration it was decided to complete what are effectively two sets of statistical analyses using

1) Median values of the four identical TMA blocks

2) All values (combat data)

The reasons for doing this were as follows:

- Median values best reflect the current practices of a hospital pathology department. When a tumour section is being assessed by the pathologist the largest area of tumour showing a particular feature – for example differentiation, tumour type, HER2 status – will determine how the tumour is classified. Any different features are noted in the pathology report but not acted on. By using only the median values, any extreme values which could potentially skew the results will be removed or not acted on.
- All values were used to take into account tumour heterogeneity. Very rarely, if ever, would a tumour show consistent features throughout, therefore it could be hypothesised that extreme values represent the histologically different areas of tumour.

3.3.2 Interpretation of statistical results

Univariate analysis was completed on both median values and combat data within the complete patient group were analysed using TMA Navigator, which is a free web-based program specifically created to analyse TMA data.[127] This was to examine each protein singly to see if it could predict survival – disease free and overall - within a patient group. The worst prognostic group in each case was selected and compared against the group of patients who received chemotherapy.

For colorectal TMAs the chemotherapy group had received 5-FU or capecitabine and for pancreatic TMAs the chemotherapy group had received gemcitabine.

Analysis on the complete patient group was completed to see if any of the proteins were predictive of survival. Analysis on the group who received chemotherapy was compared against the complete patient group to see if any proteins that predicted survival were also significant prognostically. Finally, the equation generated by Lecca et al was used to determine whether combination of proteins were predictive of resistance.[128] This formula uses the equation DCK/(RRM1 x RRM2) to generate a figure which may then be divided into high and low expression. hENT1 is

hypothetically an independent indicator of gemcitabine resistance therefore univariate analysis results were generated for this protein.

3.4 Statistical analysis for overall survival of pancreatic TMA set

3.4.1 Marker heatmaps and Spearman's correlation networks for all data

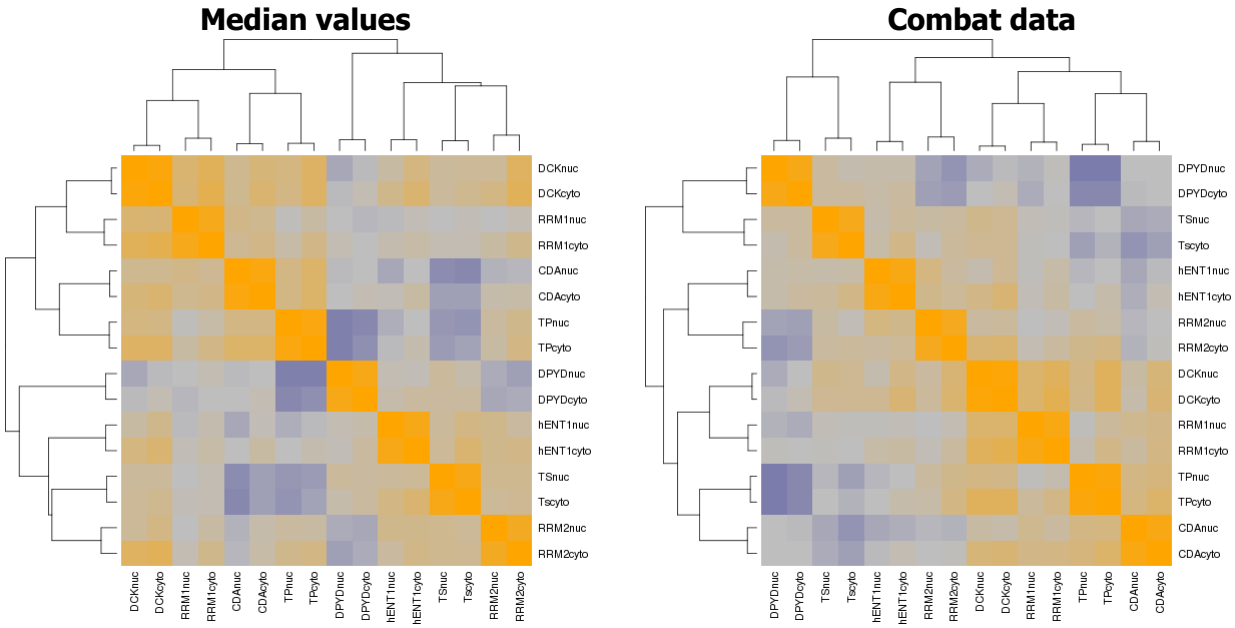


Figure 3.9 Pancreatic cancer TMA marker heatmap for median values and combat data for all patients

The above heatmaps were generated using TMA Navigator. The orange colour indicates a positive correlation and the blue colour indicates a negative correlation, any grey shades indicate no correlation. The proteins are listed along the bottom and down the right hand side.

Both median and combat values indicate that there is a strong negative correlation between TP and DPYD. There is weaker negative correlation between CDA and TP with TS and DPYD and RRM2.

The dendogram to the left and top of the diagram shows the proteins grouped together using agglomerative hierarchical clustering. This is a bottom up method which builds a hierarchy of clusters which shows relations between individual members and merges clusters of data based on similarity. [129] This is used to look at trends rather than expression levels within a dataset.

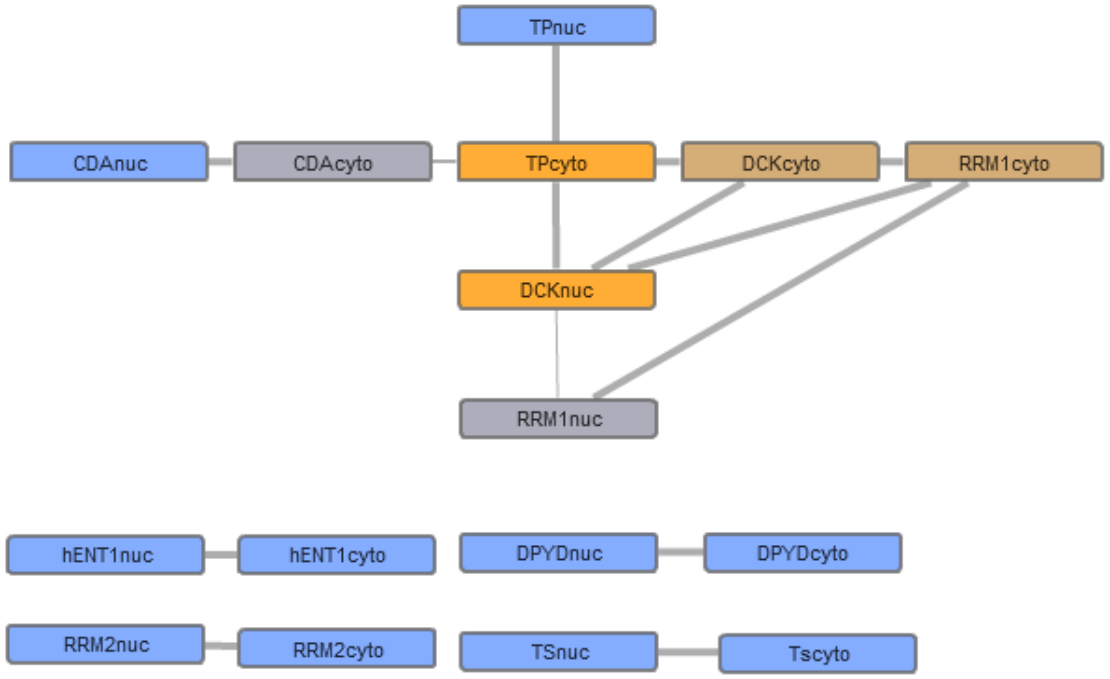


Figure 3.10 Spearman's correlation network for pancreatic cancer TMA using combat values of all patients

Spearman's correlation coefficient, or Spearman's rho, is a measure of statistical dependence between 2 variables. It determines the relationship between variables using a monotonic function; this means the values between the 2 variables increase or decrease in a similar fashion. Perfect correlation is either:

1 = strong orange shade on figures 3.8 and 3.9 or

-1 = strong blue shade on figures 3.8 and 3.9

This network sorts the 2 sets of data into ranks and tests for correlation, which means that each of the variables either increased or decreased consistently with respect to each other. The p value for this analysis was set at 0.05.

Figure 3.11 shows the correlation between the 8 proteins of interest in both nucleus and cytoplasm. A thick grey line between pairs of proteins indicates a strong correlation and a thinner grey line indicates a lesser correlation.

The table below give the Spearman's rho and p values for the pairs of proteins shown in figure 3.11.

Pair of proteins (combat data)	Spearman's rho	P value
DCKnuc – DCKcyt	0.94	0
hENT1nuc – hENT1cyt	0.88	0
CDAnuc – CDAcyt	0.88	0
RRM1nuc – RRM1cyt	0.89	0
RRM2 nuc – RRM2 cyt	0.83	0
TPnuc – TPcyt	0.92	0
DPYDnuc - DPYDcyt	0.87	0
TSnuc – TScyt	0.84	0
DCKnuc – RRM1cyt	**0.50**	**0.005**

Table 3.2 Spearman's rho and p values for pairs of proteins within pancreatic cancer TMA using combat values of all patients

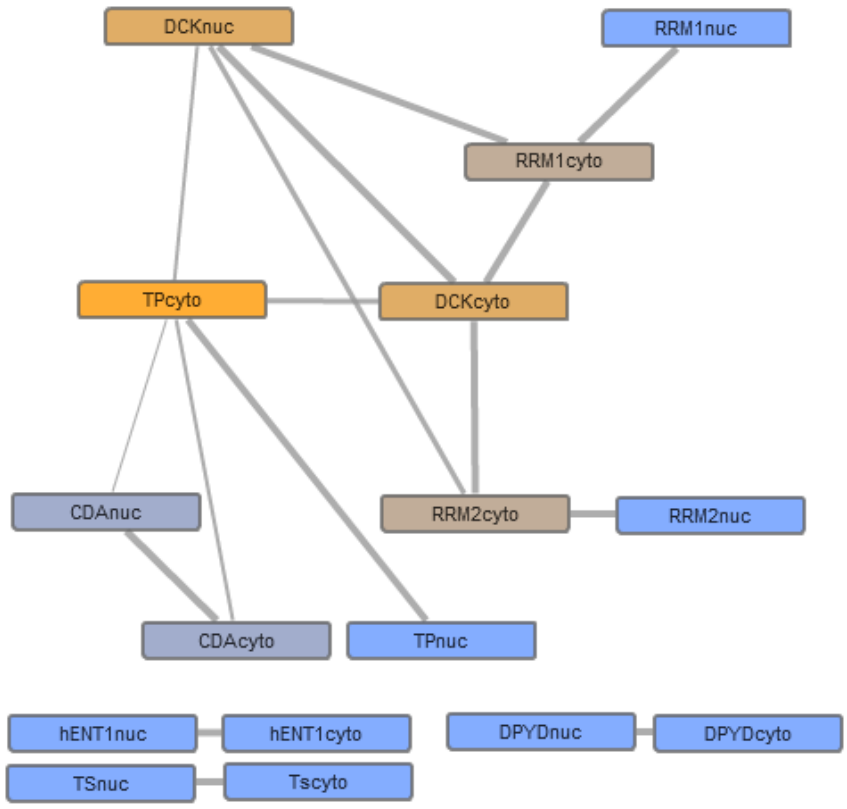


Figure 3.11 Spearman's correlation network for pancreatic cancer TMA using median data of all patients

This is the same patient set as figure 3.11 but using median values instead of all data. The table below give the Spearman's rho and p values for the pairs of proteins shown in figure 3.11.

Pair of proteins (median values)	Spearman's rho	P value
DCKnuc – DCKcyt	0.93	0
hENT1nuc – hENT1cyt	0.89	0
CDAnuc – CDAcyt	0.91	0
RRM1nuc – RRM1cyt	0.85	0
RRM2 nuc – RRM2 cyt	0.81	0
TPnuc – TPcyt	0.91	0
DPYDnuc - DPYDcyt	0.87	0
TSnuc – TScyt	0.88	0
DCKnuc – RRM1cyt	**0.53**	**0.003**
DCKnuc – RRM2cyt	**0.50**	**0.006**
DCKnuc – TPcyt	**0.47**	**0.018**
DCKcyt – RRM2cyto	**0.54**	**0.002**
TPcyt - CDAcyt	**0.46**	**0.020**
TPcyt - CDAnuc	**0.45**	**0.032**

Table 3.3 Spearman's rho and p values for pairs of proteins within pancreatic cancer TMA using median values of all patients

DCK nucleus and RRM1 cytoplasm are significantly correlated using both median values and combat data. DCK nucleus is also linked with RRM2 cytoplasm and TP cytoplasm. DCK cytoplasm is linked with RRM2 cytoplasm. TP cytoplasm is linked with CDA cytoplasm and nucleus.

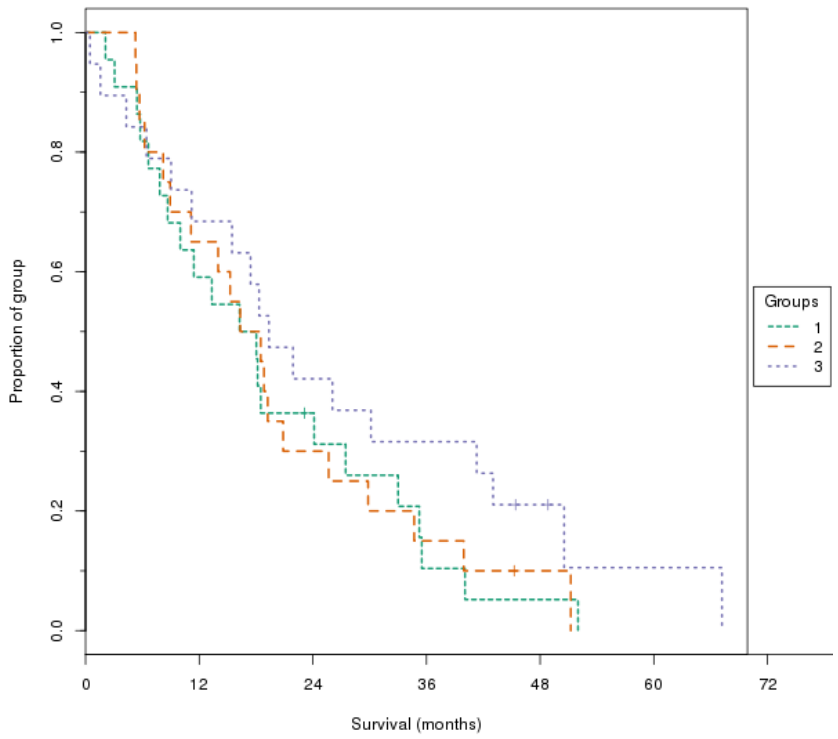
Overall and disease free survival for all cohorts of pancreatic cancer TMA can be seen in Appendix 21.

3.5 Statistical analysis using Lecca formula

3.5.1 Kaplan-Meier survival plots of all patients pancreatic TMA set

Overall survival

The graph below shows the overall survival in months of all patients in the pancreatic cancer cohort. Some of these patients will have received various chemotherapeutic treatments and some will have had no treatment. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. If we look at the survival rates we see that no patients survived past 51 months in the low and medium expression groups. All patients in the high expression group had died by 67 months. These results are not statistically significant with a p value of 0.24. Details on the Lecca formula can be found in section XX



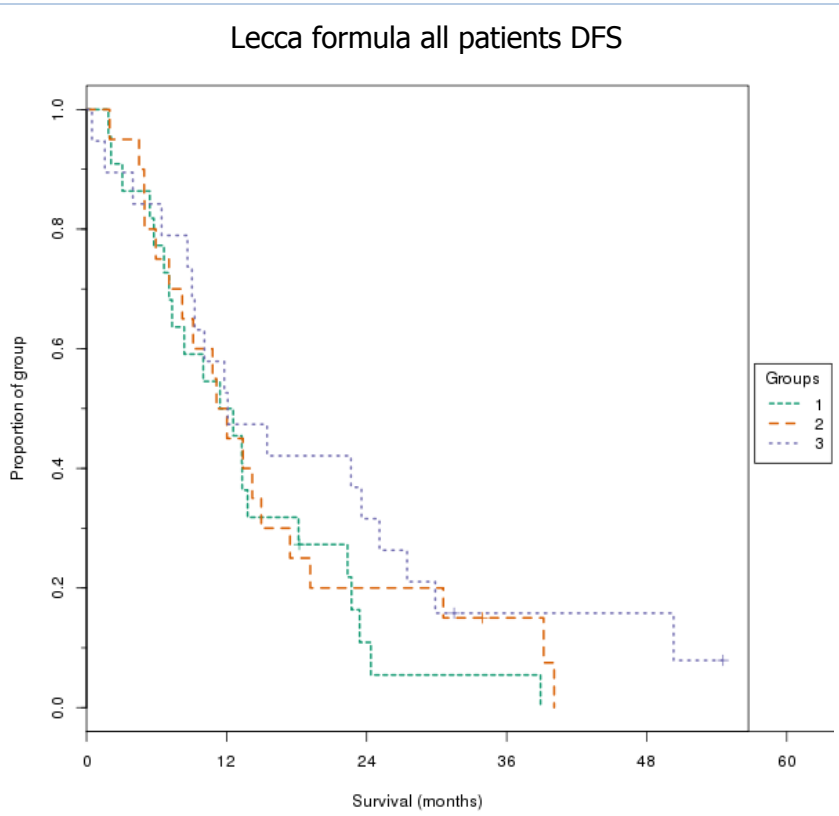
p = 0.24

Group	Expression (median)	[P value] odds ratio and 95% confidence intervals	
1(23)	L 0.00 - 236.54 (175.18)	Low-Medium	[0.26] 0.68(0.35 – 1.37)
2(22)	M 244.58 - 370.96 (302.62)	High-Medium	[0.77] 1.1 (0.59 – 2.06)
3(23)	H 376.71 - 737.81 (453.18)	Low-High	[0.16] 1.61(0.82 – 3.14)

Figure 3.12 Kaplan-Meier survival plot of pancreatic cancer TMA using Lecca formula showing overall survival of all patients: Lecca formula = DCK/(RRM1 x RRM2)

Disease free survival

The graph below shows the disease free survival in months of all patients in the pancreatic cancer cohort. Some of these patients will have received chemotherapeutic treatment and some will have had no treatment. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. All patients in the low and medium expression groups succumbed to disease progression by 39 months. 9% of patients in the high expression group were disease progression free at 58 months, however this result is not statistically significant with a p value of 0.19. Details on the Lecca formula can be found in section XX



p = 0.19

Group	Expression (median)	[P value] odds ratio and 95% confidence intervals	
1(23)	0.00 - 236.54 (175.18)	Low-Medium	[0.47] 1.27 (0.67 – 2.4)
2(22)	244.58 - 370.96 (302.62)	High-Medium	[0.39] 0.75 (0.38 – 1.46)
3(23)	376.71 - 737.81 (453.18)	Low-High	[0.12] 1.7 (0.87 – 3.33)

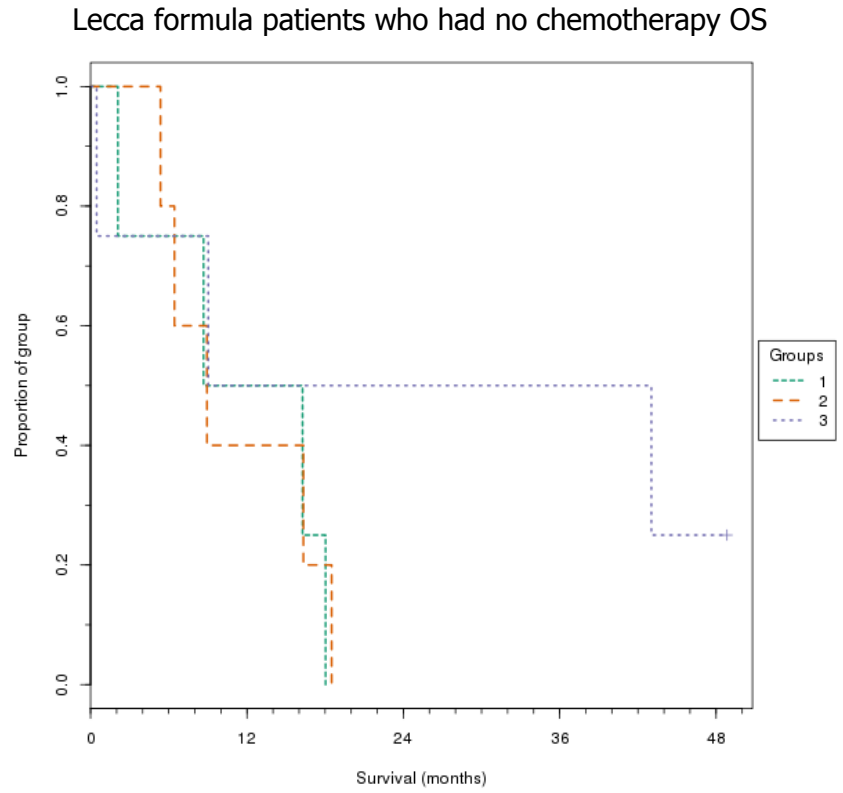
Figure 3.13 Kaplan-Meier survival plot of pancreatic cancer TMA using Lecca formula showing disease free survival of all patients: Lecca formula = DCK/(RRM1 x RRM2)

3.5.2 Kaplan-Meier survival plots of patients who had no chemotherapy pancreatic TMA set

Overall survival

The graph below shows the overall survival in months of the patients in the pancreatic cancer cohort who did not undergo any chemotherapeutic treatment. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The low and medium expression groups survival lines are almost identical with no patients surviving past 19 months. This compares to the high expression group where 25% of patients were still alive at 48 months. This result is not statistically significant with a p value of 0.24.

Details on the Lecca formula can be found in section 2.3.5.1.



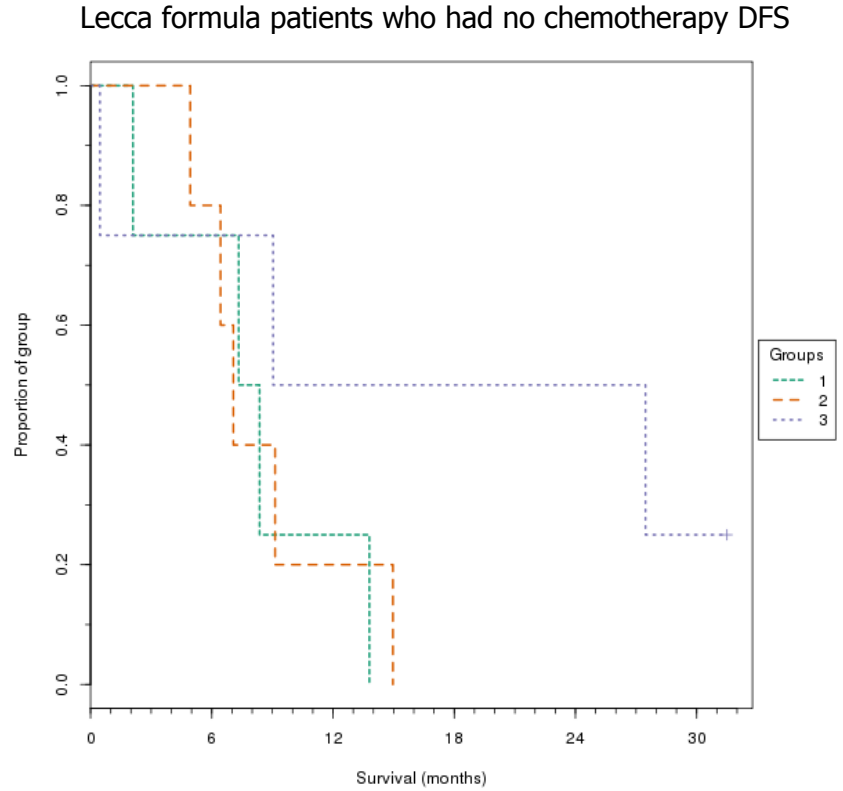
p = 0.24

Group (n = 5)	Expression (median)	[P value] odds ratio and 95% confidence intervals	
1	106.08 - 280.86 (188.49)	Low-Medium	[0.84] 1.15 (0.3 – 4.39)
2	281.29 - 412.44 (328.39)	High-Medium	[0.23] 2.75 (0.52 – 14.53)
3	433.30 - 721.68 (514.04)	Low-High	[0.2] 3.15 (0.54 – 18.41)

Figure 3.14 Kaplan-Meier survival plot of pancreatic cancer TMA using Lecca formula showing overall survival of patients who had no chemotherapy: Lecca formula = DCK/(RRM1 x RRM2)

Disease free survival

The graph below shows the disease free survival in months of the patients in the pancreatic cancer cohort who did not undergo any chemotherapeutic treatment. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The low and medium expression survival lines are almost identical with all patients showing disease progression at 15 and 18 months respectively. This compares to the high expression group where 25% of patients were progression free at 30 months. This result is not statistically significant with a p value of 0.20. Details on the Lecca formula can be found in section 2.3.5.1.



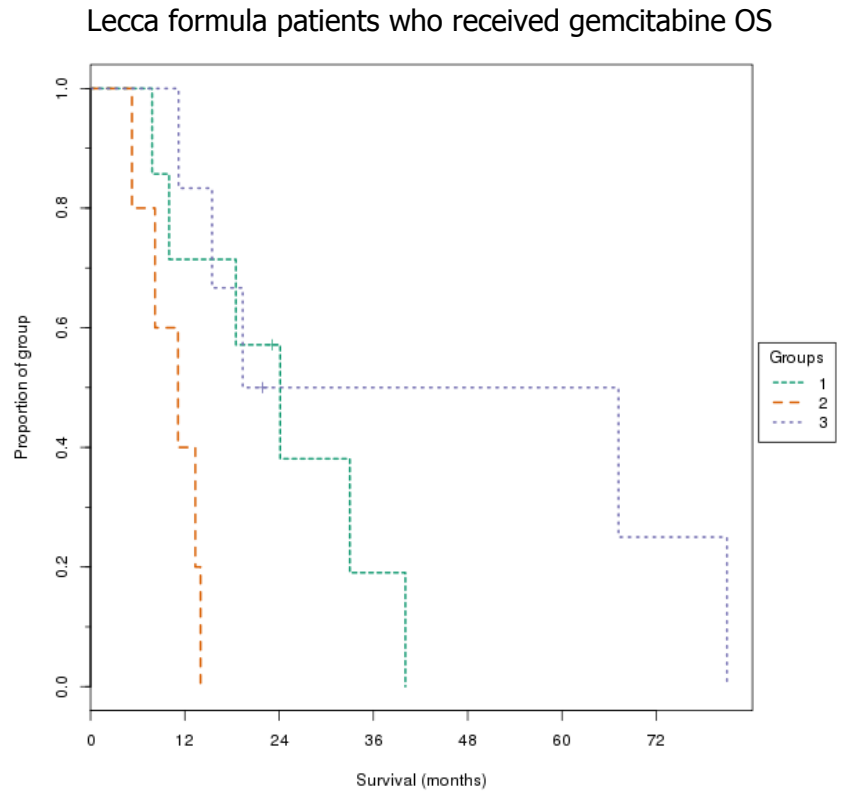
p = 0.20			
Group (n = 5)	**Expression (median)**	**[P value] odds ratio and 95% confidence intervals**	
1	106.08 - 280.86 (188.49)	Low-Medium	[0.86] 1.13 (0.29 – 4.38)
2	281.29 - 412.44 (328.39)	High-Medium	[0.2] 2.96 (0.56 – 15.6)
3	433.30 - 721.68 (514.04)	Low-High	[0.18] 3.35 (0.57 – 19.6)

Figure 3.15 Kaplan-Meier survival plot of pancreatic cancer TMA using Lecca formula showing disease free survival of patients who had no chemotherapy: Lecca formula = DCK/(RRM1 x RRM2)

3.5.3 Kaplan-Meier survival plots of patients who received gemcitabine pancreatic TMA set

Overall survival

The graph below shows the overall survival in months of the patients in the pancreatic cancer cohort who received gemcitabine. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The longest survival time of the low expression group was 14 months, compared to 39 months for the medium expression group and 80 months for the high expression group. This result is statistically significant with a p value of 0.007. Details on the Lecca formula can be found in section 2.3.5.1.



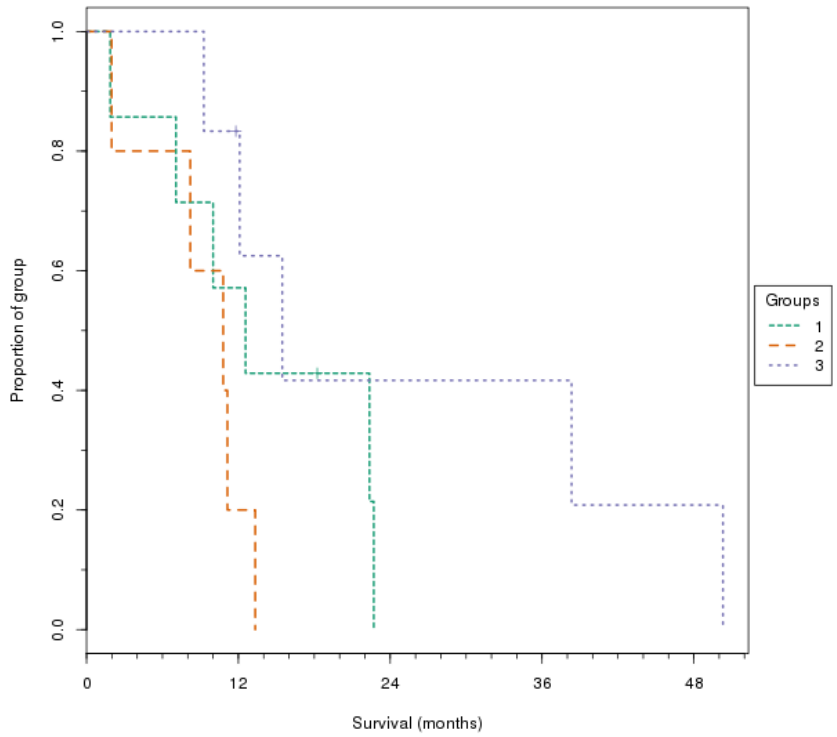
p = 0.007			
Group (n=7)	**Expression (median)**	**[P value] odds ratio and 95% confidence intervals**	
1	0.00 - 212.70 (191.22)	Low-Medium	[0.05] 0.216 (0.05 – 1.02)
2	236.54 - 310.16 (288.98)	High-Medium	[0.009] 9.77 (1.75 – 54.4)
3	317.70 - 737.81 (388.77)	Low-High	[0.31] 2.11 (0.5 – 8.9)

Figure 3.16 Kaplan-Meier survival plot of pancreatic cancer TMA using Lecca formula showing overall survival of patients who received gemcitabine: Lecca formula = DCK/(RRM1 x RRM2)

Disease free survival

The graph below shows the disease free survival in months of the patients in the pancreatic cancer cohort who received gemcitabine. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The low, medium and high expression groups showed all patients having succumbed to disease progression at 23, 13 and 50 months respectively. This result is not statistically significant with a p value of 0.07.

Details on the Lecca formula can be found in section 2.3.5.1.



p = 0.07			
Group (n=7)	**Expression (median)**	**[P value] odds ratio and 95% confidence intervals**	
1	0.00 - 212.70 (191.22)	Low-Medium	[0.37] 0.52 (0.14 – 1.95)
2	236.54 - 310.16 (288.98)	High-Medium	[0.05] 4.52 (0.99 – 20.6)
3	317.70 - 737.81 (388.77)	Low-High	[0.24] 2.37 (0.56 – 9.98)

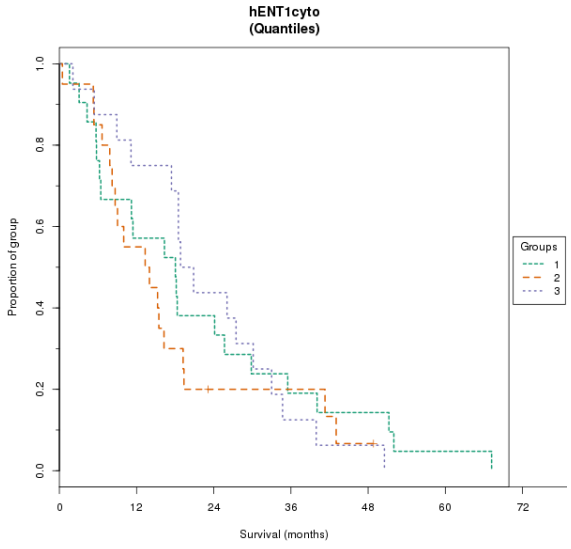
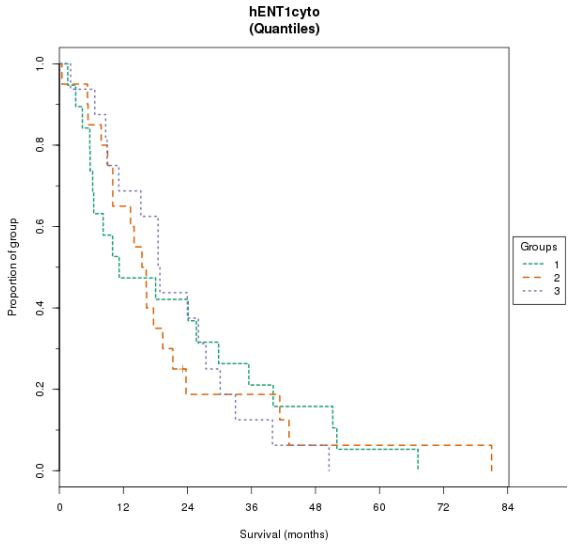
Figure 3.17 Kaplan-Meier survival plot of pancreatic cancer TMA using Lecca formula showing disease free survival of patients who received gemcitabine: Lecca formula = DCK/(RRM1 x RRM2)

3.6 Statistical analysis of cytoplasm hENT1

3.6.1 Kaplan-Meier survival plots of all patients pancreatic cancer TMA set

The graphs below show the overall survival plotted against cytoplasmic expression of hENT1 for all pancreatic cancer patients in this cohort. Some of these patients would have received chemotherapeutic intervention and some would have no treatment. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.[104] If the total tumour area is less than 5% of the total area of the spot then it is automatically excluded from analysis.

Comparing the graphs, there is little difference between median and combat analysis and there is no definitive separation of the groups. In the median values graph the low expression group survived longest whereas in the combat values graph the medium expression group survived longest. The p values are 0.37 and 0.55 for median values and combat values respectively. None of these are statistically significant.

Median values	Combat data
	
p = 0.37	**p = 0.55**

Group	Expression	Median value	Group (n=26)	Expression	Median value
1 (25)	77.33 – 186.96	142.01	1	87.89 - 187.82	141.75
2 (24)	187.82 – 251.22	229.68	2	191.55 - 254.57	226.27
3 (24)	254.57 – 1254.99	475.33	3	254.90 - 1625.25	451.38
[P value] odds ratio and 95% confidence intervals					
Low-Medium	[0.11] 0.58 (0.3 – 1.14)				
High-Medium	[0.23] 0.67 (0.34 – 1.29)				
Low-High	[0.68] 0.88 (0.46 – 1.66)				

Figure 3.18 Kaplan-Meier (tertile) plots of pancreatic cancer TMA hENT1 cytoplasm expression showing proportion of group against overall survival in months (median values versus combat data all patients)

The graphs below show the disease free survival plotted against cytoplasmic expression of hENT1 for all pancreatic cancer patients in this cohort. Some of these patients would have received chemotherapeutic intervention and some would have no treatment. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.[104] If the total tumour area is less than 5% of the total area of the spot then it is automatically excluded from analysis.

Comparing the graphs, the low and medium expression groups have almost identical times to disease progression when compared with the high expression group. The median and combat values are very similar with the high expression group in both graphs having a slightly longer time to disease progression than the low and medium expression groups. The p values are 0.35 and 0.5 for median values and combat values respectively. None of these p values are statistically significant.

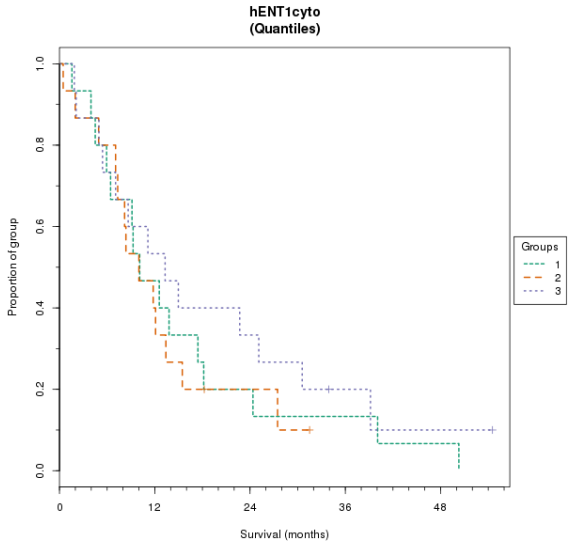
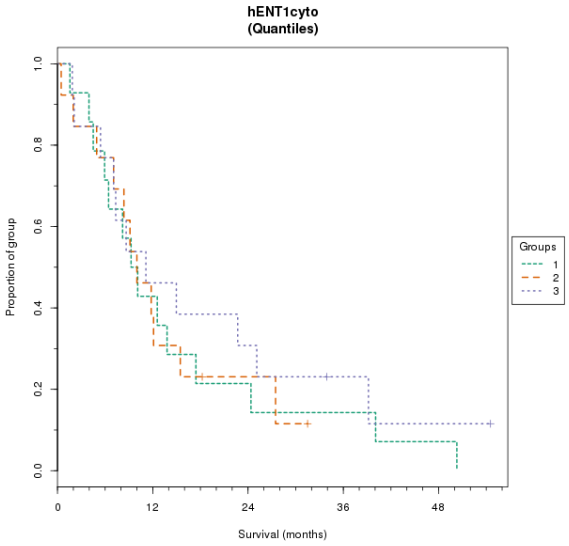
Median values			Combat data		
					
p = 0.35			**p = 0.5**		
Group	**Expression**	**Median value**	**Group (n=26)**	**Expression**	**Median value**
1(25)	77.33 - 186.96	142.01	1	87.89 - 187.82	141.75
2(24)	187.82 - 251.22	229.68	2	191.55 - 254.57	226.27
3(24)	254.57 - 1254.99	475.33	3	254.90 - 1625.25	451.38
[P value] odds ratio and 95% confidence intervals					
Low-Medium	[0.54] 0.78 (0.36 – 1.69)				
High-Medium	[0.33] 0.68 (0.31 – 1.47)				
Low-High	[0.68] 1.16 (0.57 – 2.35)				

Figure 3.19 Kaplan-Meier (tertile) plots of pancreatic cancer TMA hENT1 cytoplasm expression showing proportion of group against disease free survival in months (median values versus combat data all patients)

3.6.2 Kaplan-Meier survival plots of patients who received gemcitabine pancreatic cancer TMA set

The graphs below show the overall survival plotted against cytoplasmic expression of hENT1 for all pancreatic cancer patients who received gemcitabine. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total

tumour area and light exposure time.[104] If the total tumour area is less than 5% of the total area of the spot then it is automatically excluded from analysis.

The medium and high expression groups are similar in both graphs and the low expression groups fare best with the last patient surviving 67 months.

This is a direct contradiction to our hypothesis which predicts that high hENT1 levels are indicative of gemcitabine sensitivity. The p values are 0.29 and 0.2 for median values and combat values respectively. None of these p values are statistically significant.

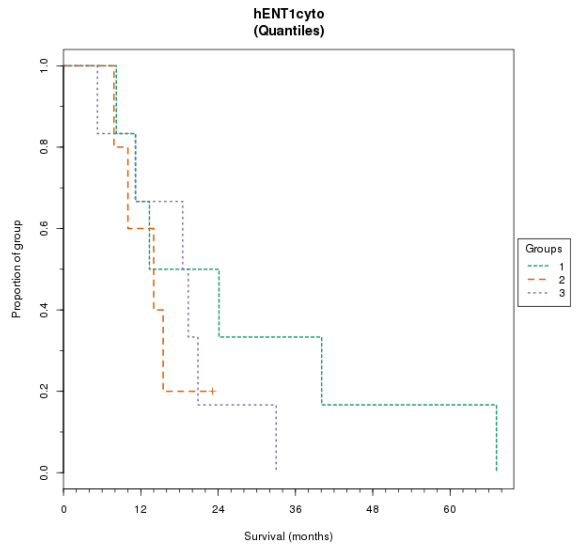
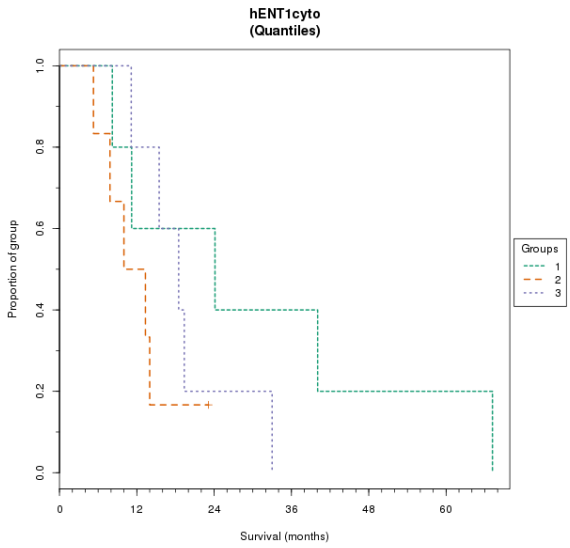
Median values			Combat data		
					
p = 0.29			**p = 0.2**		
Group (n=8)	**Expression**	**Median value**	**Group**	**Expression**	**Median value**
1	93.80 – 198.11	184.83	1(8)	93.80 - 197.02	169.23
2	198.42 – 235.22	214.56	2(7)	198.11 - 229.59	218.41
3	236.32 - 563.56	252.39	3(7)	230.71 - 1625.25	276.40
[P value] odds ratio and 95% confidence intervals					
Low-Medium	[0.83] 1.17 (0.28 – 4.98)				
High-Medium	[0.31] 0.48 (0.11 – 1.98)				
Low-High	[0.36] 0.56 (0.16 – 1.94)				

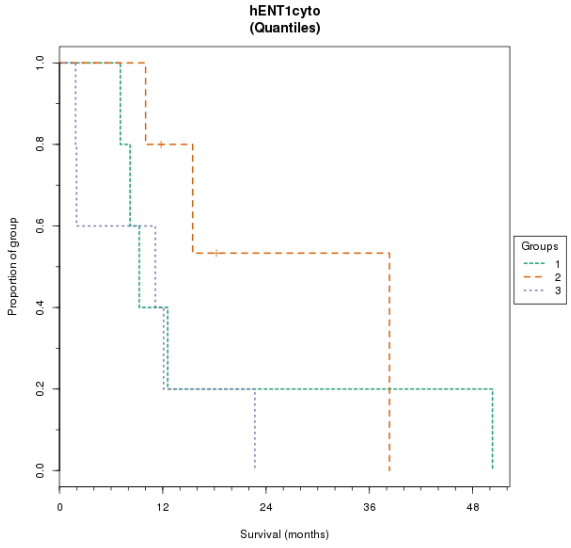
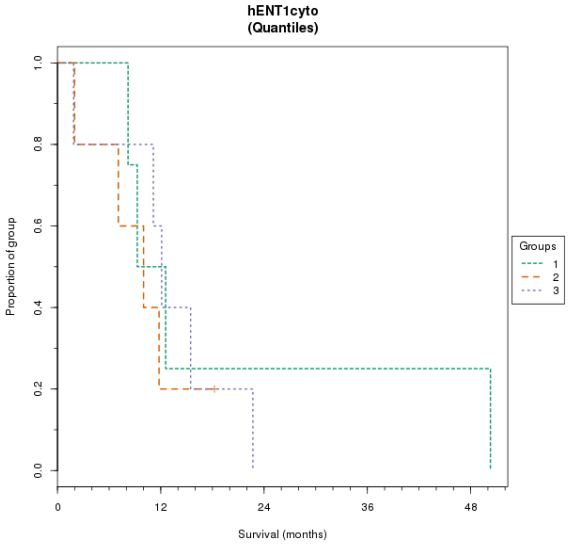
Figure 3.20 Kaplan-Meier (tertile) plots of pancreatic cancer TMA hENT1 cytoplasm expression showing proportion of group against overall survival in months (median values versus combat data all patients that received gemcitabine)

The graphs below show the disease free survival plotted against cytoplasmic expression of hENT1 for all pancreatic cancer patients who received gemcitabine. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.[104] If the total tumour area is less than 5% of the total area of the spot then it is automatically excluded from analysis.

The median values graph shows separation of the groups with the low expression group taking the longest time to disease progression, however the p value is 0.17.

The combat values graph shows the 3 expression groups are almost identical with the low expression taking the longest time to disease progression, however this isn't statistically significant with a p value of 0.62.

This is a direct contradiction to our hypothesis which predicts that high hENT1 levels are indicative of longer survival.

Median values	**Combat data**
	
p = 0.17	**p = 0.62**

Group (n=7)	**Expression**	**Median value**	**Group**	**Expression**	**Median value**
1	118.30 – 199.45	185.10	1(8)	93.80 - 197.02	169.23
2	200.00 - 235.22	228.16	2(7)	198.11 - 229.59	218.41
3	236.32 - 563.56	280.00	3(7)	230.71 - 1625.25	276.40

[P value] odds ratio and 95% confidence intervals	
Low-Medium	[0.5] 0.54 (0.09 – 3.28)
High-Medium	[0.23] 2.49 (0.56 – 10.96)
Low-High	[0.09] 0.22 (0.04 – 1.24)

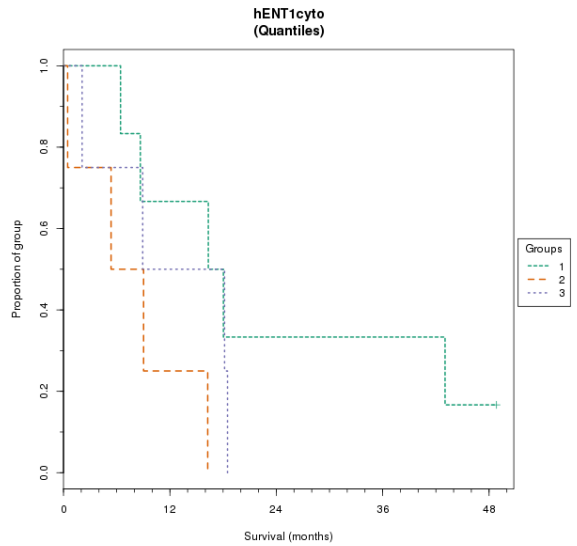
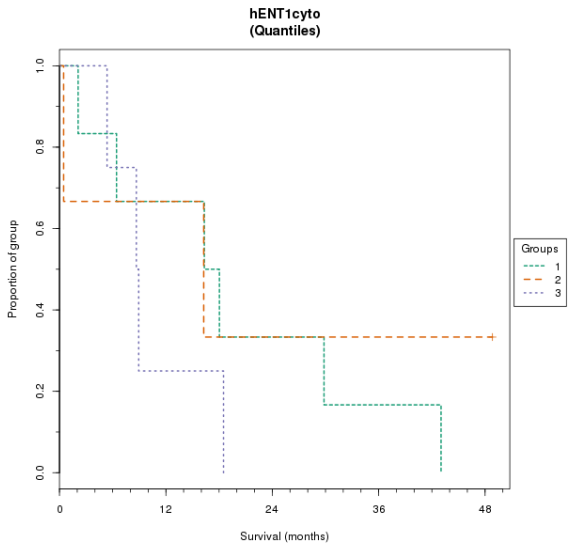
Figure 3.21 Kaplan-Meier (tertile) plots of pancreatic cancer TMA hENT1 cytoplasm expression showing proportion of group against disease free survival in months (median values versus combat data all patients that received gemcitabine)

3.6.3 Kaplan-Meier survival plots of patients who had no chemotherapy pancreatic cancer TMA set

The graphs below show the overall survival plotted against cytoplasmic expression of hENT1 for all pancreatic cancer patients who did not receive chemotherapy. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.[104] If the total tumour area is less than 5% of the total area of the spot then it is automatically excluded from analysis.

In the median values graph the medium and high expression groups have a longest survival time of 16 and 18 months respectively. The low expression group fared best with the longest survival time of greater than 48 months.

In the combat values graph the high and low expression groups have a longest survival time of 18 and 44 months. The medium expression group has a longest survival time of more than 48 months. The p values are 0.13 and 0.47 for median values and combat values respectively. None of these p values are statistically significant.

Median values	Combat data
	

p = 0.13			p = 0.47		
Median values			**Combat data**		
Group (n=6)	**Expression**	**Median value**	**Group**	**Expression**	**Median value**
1	138.52 - 220.35	191.55	1(6)	131.30 - 191.55	154.80
2	231.27 - 244.84	236.68	2(5)	206.09 - 258.59	244.84
3	254.57 - 1020.48	340.65	3(5)	261.81 - 1088.83	334.67
[P value] odds ratio and 95% confidence intervals					
Low-Medium	[0.26] 0.49 (0.15 – 1.69)				
High-Medium	[0.37] 0.59 (0.18 – 1.87)				
Low-High	[0.79] 0.85 (0.25 – 2.82)				

Figure 3.22 Kaplan-Meier (tertile) plots of pancreatic cancer TMA hENT1 cytoplasm expression showing proportion of group against overall survival in months (median values versus combat data all patients that did not receive chemotherapy)

The graphs below show the disease free survival plotted against cytoplasmic expression of hENT1 for all pancreatic cancer patients who did not receive chemotherapy. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.[104] If the total tumour area is less than 5% of the total area of the spot then it is automatically excluded from analysis.

In the median values graph the low expression groups has the shortest time to disease progression – 8 months. The medium and high expression groups both have approximately 20% of the group progression free at 30 months. This is not statistically significant with a p value of 0.09.

The combat values graph shows the low and high expression groups with near identical lines with 20% of both groups progression free at 24 months. The medium expression group has 35% of patients progression free at 30 months. The combat values graph is not statistically significant with a p value of 0.67.

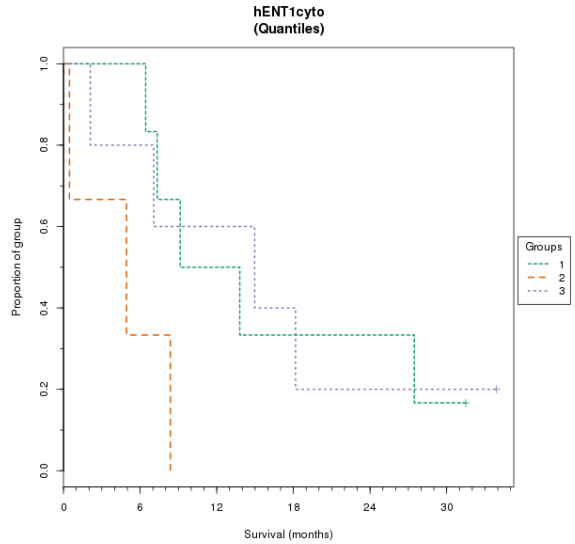
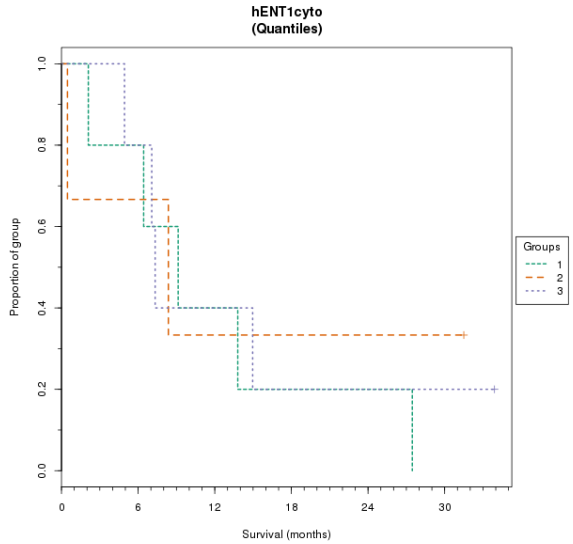
Median values			Combat data		
					
Median values			**Combat data**		
p = 0.09			**p = 0.67**		
Group	**Expression**	**Median value**	**Group**	**Expression**	**Median value**
1(7)	138.52 - 220.35	191.55	1(6)	131.30 - 191.55	154.80
2(6)	231.27 - 244.84	236.68	2(5)	206.09 - 258.59	244.84
3(6)	254.57 - 1020.48	340.65	3(5)	261.81 - 1088.83	334.67
[P value] odds ratio and 95% confidence intervals					
Low-Medium	[0.16] 0.42 (0.12 – 1.43)				
High-Medium	[0.27] 0.52 (0.16 – 1.65)				
Low-High	[0.73] 0.81 (0.24 – 2.69)				

Figure 3.23 Kaplan-Meier (tertile) plots of pancreatic cancer TMA hENT1 cytoplasm expression showing proportion of group against disease free survival in months (median values versus combat data all patients that did not receive chemotherapy)

3.7 Colorectal cancer TMA set results

All graphs can be seen in Appendix 22.

The graphs show analysis of the 5 proteins involved in the metabolism of 5-FU to its active state – TP, DPYD, TS, RRM1 and RRM2. The cohort was divided into Dukes A/B and Dukes C/D.

The Kaplan Meier survival graphs show the overall and disease free survival for

a) All colorectal cancer patients regardless of whether or not they received treatment.

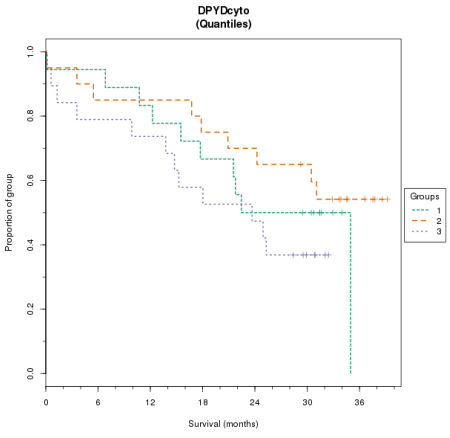
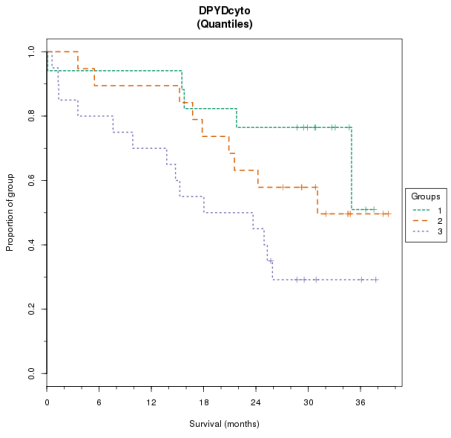
b) Patients who received 5-FU.

c) Patients who did not receive chemotherapy.

3.7.1 Kaplan-Meier survival plots colorectal cancer TMA set of all patients

The only significant result is DPYD in the Dukes C/D cohort. Low expression levels of DPYD (in Dukes C/D) are linked to longer overall and disease free survival, the p values are 0.03 and 0.07 for the survival types respectively. What is interesting about this result is that the AQUA expression levels range from 31.28 to 84.96. If conventional IHC had been used on these sections they would have been scored negative. However, it would appear that even extremely low scoring is important clinically with regards to DPYD.

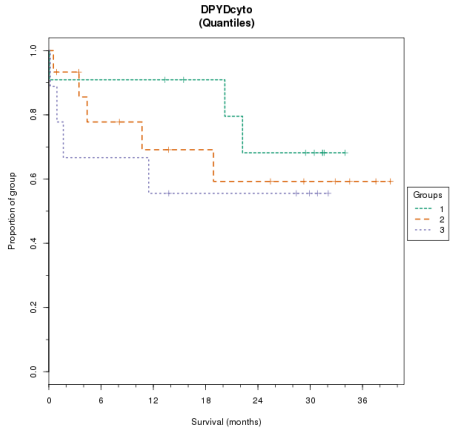
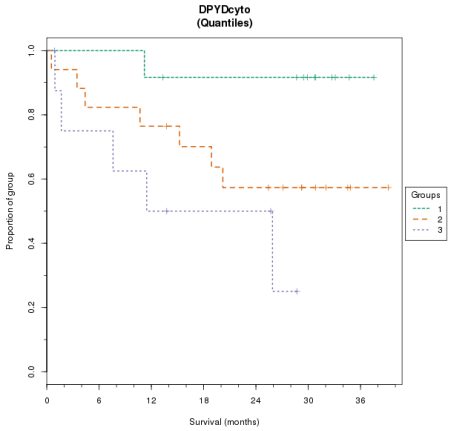
Overall survival Dukes C/D cytoplasmic expression of DPYD

Median values	Combat data
	
p = 0.36	**p = 0.03**

Group	Expression	Median value	Group (n=21)	Expression	Median value
1 (21)	31.28 - 37.48	34.30	1	32.14 - 38.37	36.80
2 (20)	37.72 - 43.14	40.87	2	39.02 - 44.14	41.76
3 (21)	43.60 - 84.96	46.69	3	44.16 - 84.96	46.99

Figure 3.24 Kaplan-Meier (tertile) plots of Dukes C/D colorectal cancer TMA DPYD cytoplasm expression showing proportion of group against overall survival in months (median values versus combat data all patients)

Disease free survival Dukes C/D cytoplasmic expression of DPYD

Median values	Combat data
	
p = 0.61	**p = 0.07**

Group	Expression	Median value	Group (n=21)	Expression	Median value
1 (21)	31.28 - 37.48	34.30	1	32.14 - 38.37	36.80
2 (20)	37.72 - 43.14	40.87	2	39.02 - 44.14	41.76
3 (21)	43.60 - 84.96	46.69	3	44.16 - 84.96	46.99

Figure 3.25 Kaplan-Meier (tertile) plots of Dukes C/D colorectal cancer TMA DPYD cytoplasm expression showing proportion of group against disease free survival in months (median values versus combat data all patients)

3.7.2 Kaplan-Meier survival plots colorectal cancer TMA set of all patients who received 5-FU

All graphs can be seen in Appendix 22.

The Kaplan Meier survival graphs below show the overall and disease free survival for all colorectal cancer patients who received 5-FU either singly or as a combination therapy.

The only significant result is DPYD in the Dukes C/D cohort. Low expression levels of DPYD (in Dukes C/D) are linked to longer overall survival, the p value is 0.029. What is interesting about this result is that the AQUA expression levels range from 32.14 to 47.54. If conventional IHC had been used on these sections they would have been scored negative. However, it would appear that even extremely low scoring is important clinically with regards to DPYD.

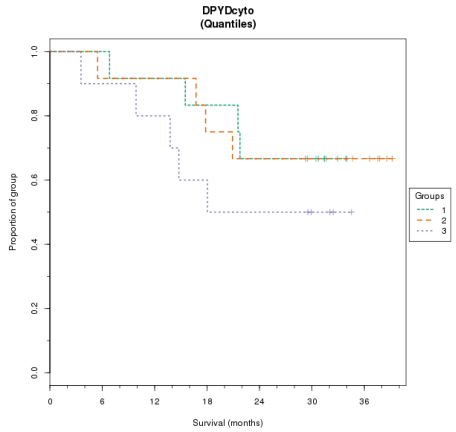
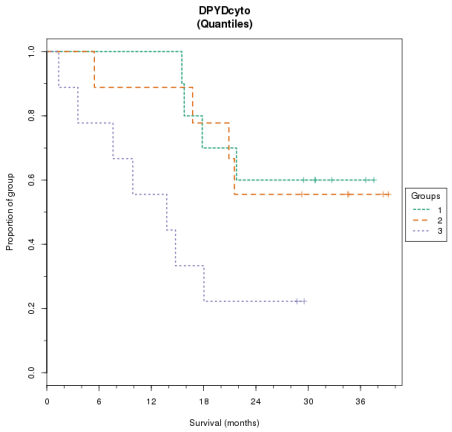
Median values			Combat data		
					
p = 0.45			**p = 0.029**		
Group (n=12)	**Expression**	**Median value**	**Group**	**Expression**	**Median value**
1	31.35 - 37.48	34.25	1 (10)	32.14 - 39.02	36.58
2	39.02 - 42.94	41.00	2 (9)	39.50 - 44.14	41.76
3	43.14 - 53.92	48.16	3 (10)	44.29 - 57.70	47.54

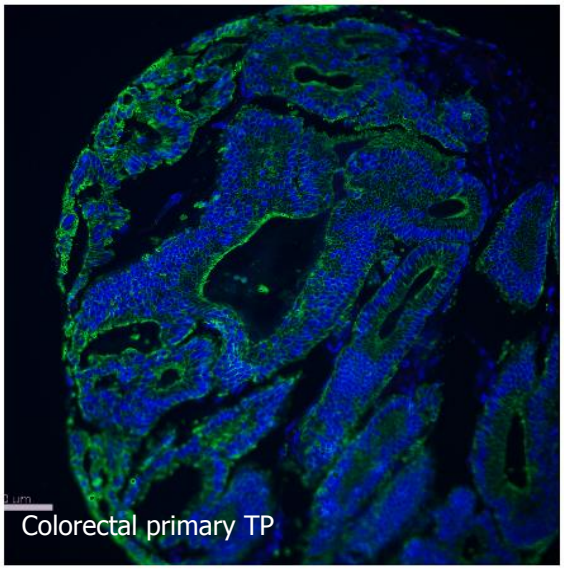
Figure 3.26 Kaplan-Meier (tertile) plots of Dukes C/D colorectal cancer TMA DPYD cytoplasm expression showing proportion of group against overall survival in months (median values versus combat data all patients who received 5-FU)

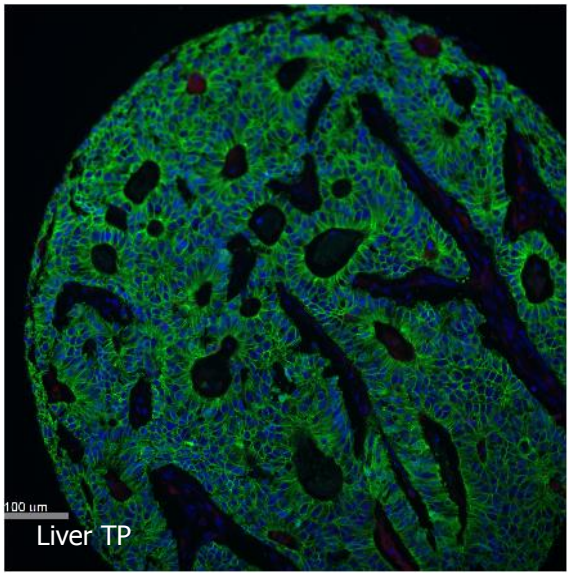
3.7.3 Kaplan-Meier survival plots colorectal cancer TMA set of all patients who did not receive chemotherapy

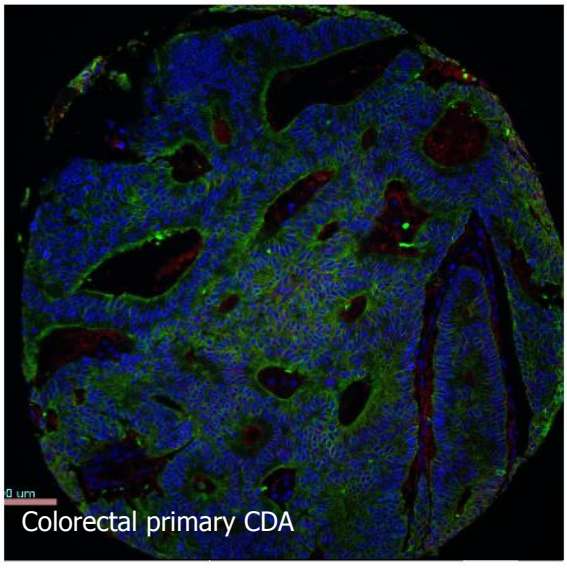
All graphs can be seen in Appendix 22. There are no statistically significant results for this section.

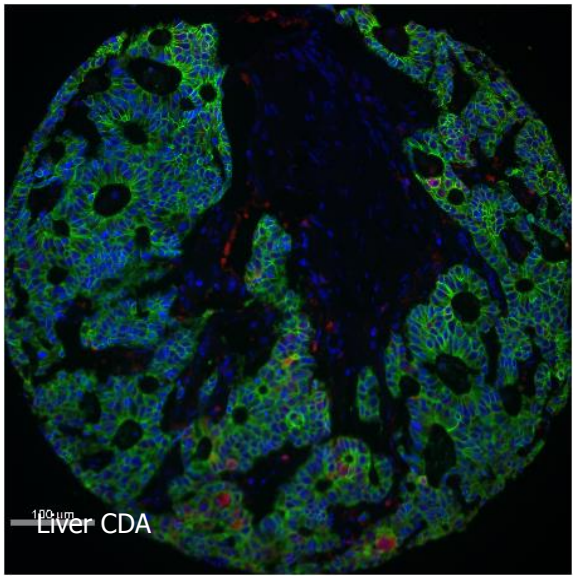
3.8 Colorectal cancer primary and matched liver metastases results

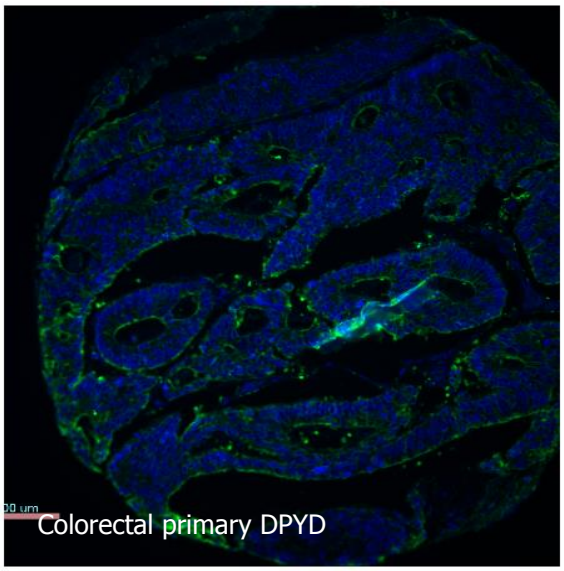
The pictures below show a primary colorectal tumour on the left with the liver metastasis on the right. The proteins shown are linked with gemcitabine metabolism – hENT1, DCK, RRM1 and RRM2 – and also those linked with 5-FU metabolism – TS, TP, RRM1, RRM2 and DPYD. The intention is to show how protein expression can change during metastasis.

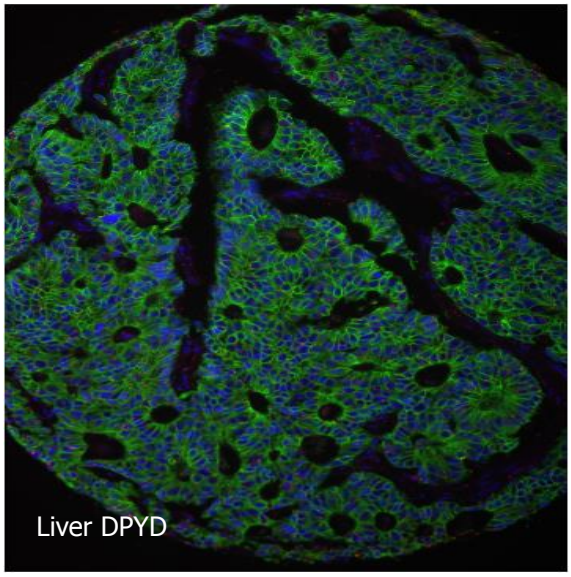


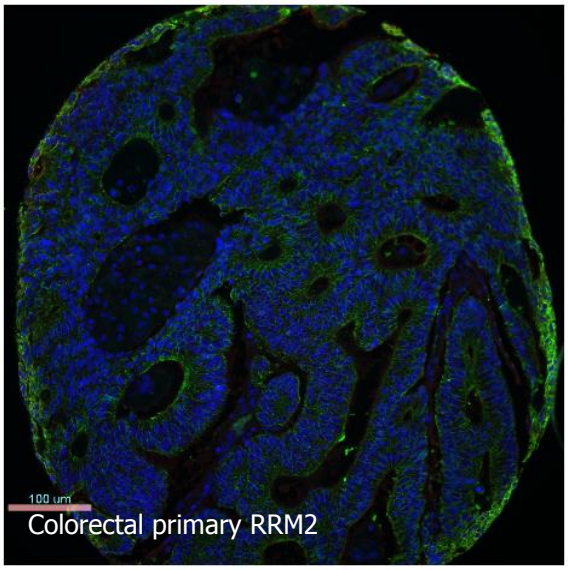


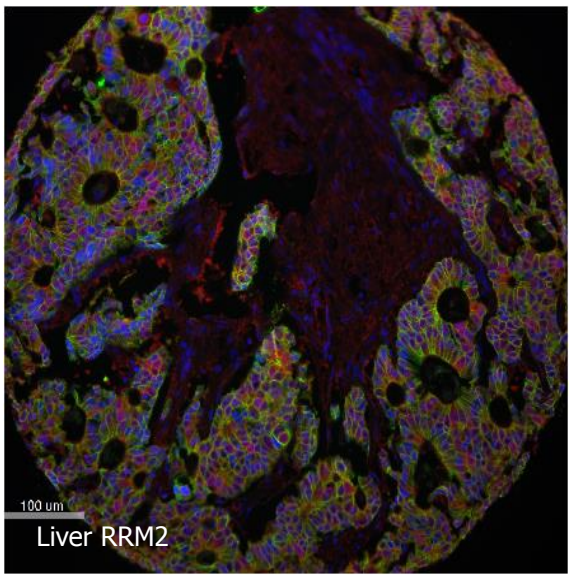


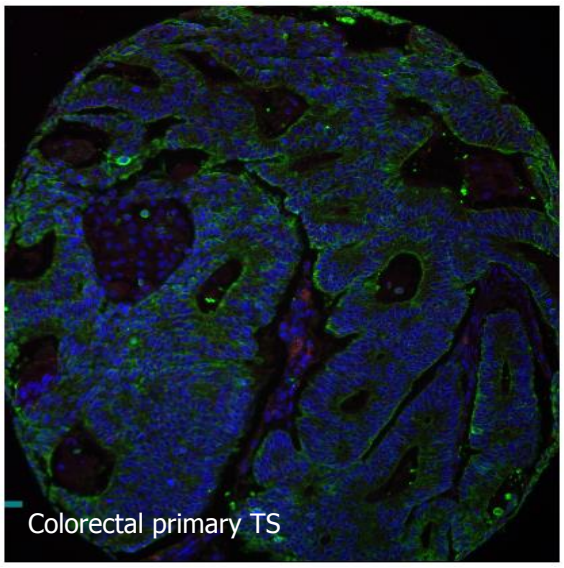


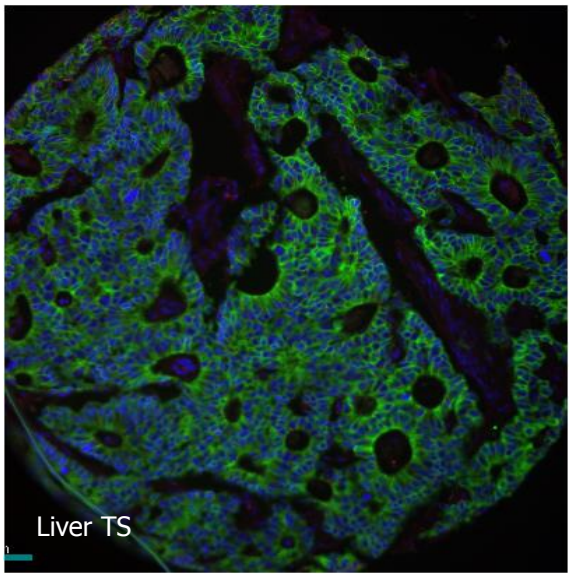

Colorectal primary DPYD


Liver DPYD


100 um
Colorectal primary RRM2


100 um
Liver RRM2


Colorectal primary TS


Liver TS

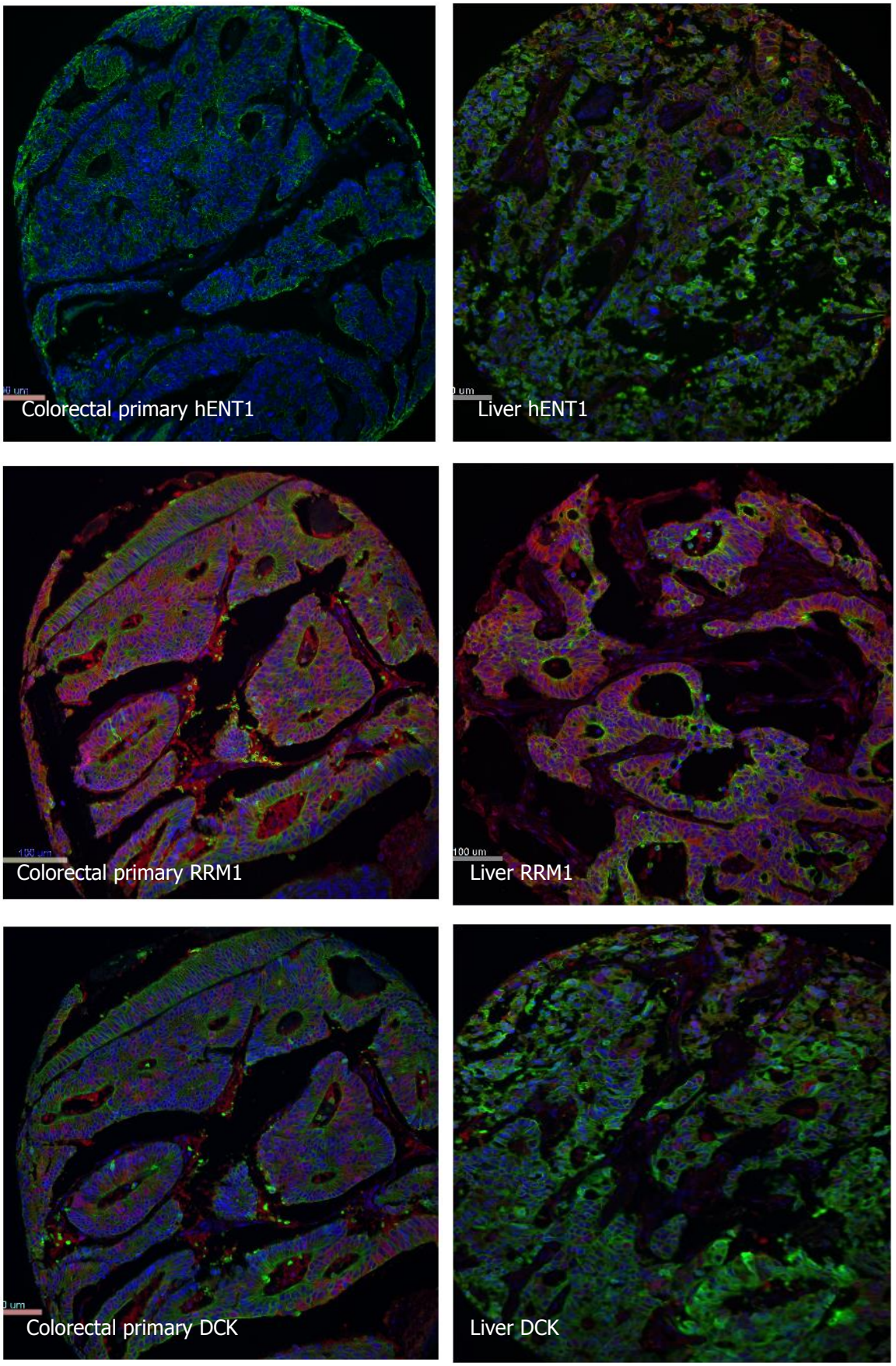


Figure 3.27 Matched colorectal cancer primary tumour on the left and matched liver metastasis on the right showing the change in protein expression that may occur during metastasis

Statistical analysis of this TMA set of patients showed that the protein RRM2 was important in all patient groups. The results below show the overall survival results for all patients RRM2 cytoplasm expression. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.[104] These results show that high RRM2 expression is linked to longer overall survival.

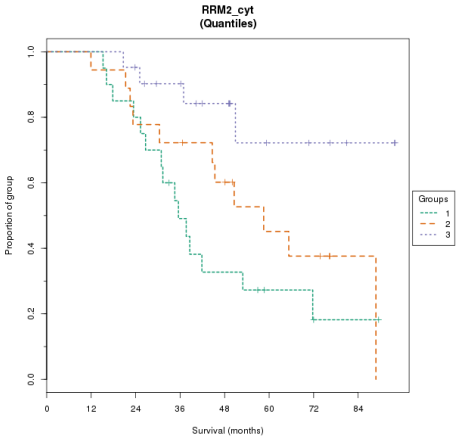
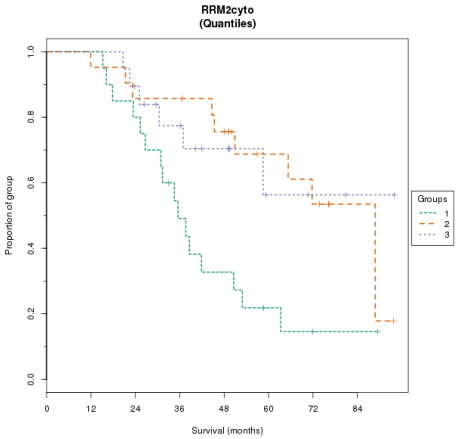
Median values			Combat data		
					
p = 0.026			**p = 0.038**		
Group (n=26)	**Expression**	**Median value**	**Group**	**Expression**	**Median value**
1	1023.24 - 2072.37	1587.65	1 (27)	105.96 - 2037.72	1568.19
2	2073.89 - 3111.38	2406.80	2 (26)	2073.89 - 3445.94	2524.08
3	3247.30 - 12468.20	4277.32	3 (26)	3717.51 - 12468.20	4800.11

Figure 3.28 Kaplan-Meier (tertile) plots of colorectal primary cancer TMA RRM2 cytoplasm expression showing proportion of group against overall survival in months (median values versus combat data all patients)

The results below show the overall and disease free survival results for all patients who received 5-FU RRM2 cytoplasm expression. The analysis was run using TMA Navigator, a statistical programme which automatically divides the cohort into three equal groups. The TMAs were constructed in quadruplicate – the data from all four TMAs was used for combat analysis and the median value of the 4 TMAs was used for the median analysis. These two analyses were undertaken to see if the two methods of analysis produced conflicting results. The expression, or AQUA score, is determined by the pixel intensity (or fluorescence) of the target protein in the cytoplasm or nucleus (or both). This value is normalised by calculating the total tumour area and light exposure time.[104]

High RRM2 expression is linked to longer overall survival and surprisingly, medium and high RRM2 expression are linked to longer disease free time.

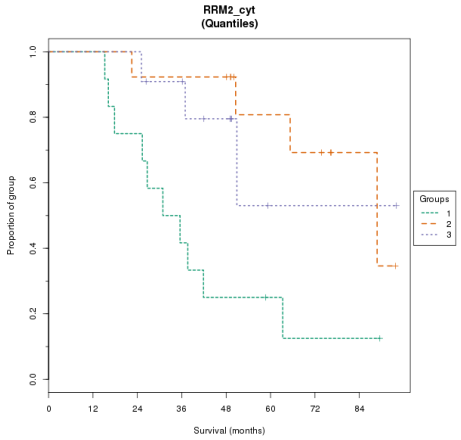
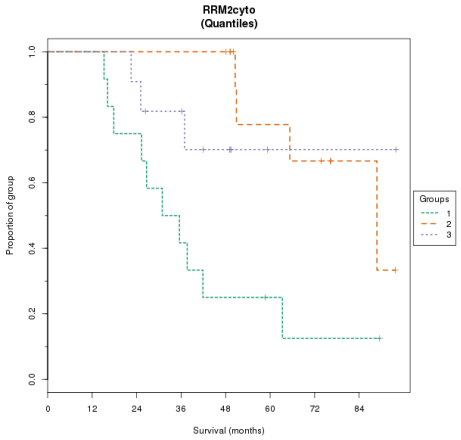
Median values			Combat data		
					
p = 0.012			**p = 0.011**		
Group (n=15)	**Expression**	**Median value**	**Group (n=15)**	**Expression**	**Median value**
1	105.96 - 1728.37	1376.24	1	105.96 - 1913.75	1467.90
2	2073.89 - 3445.94	2577.97	2	2037.72 - 3445.94	2577.97
3	3495.84 - 12468.20	4283.77	3	3717.51 - 12468.20	4653.50

Figure 3.29 Kaplan-Meier (tertile) plots of colorectal primary cancer TMA RRM2 cytoplasm expression showing proportion of group against overall survival in months (median values versus combat data all patients who received 5-FU)

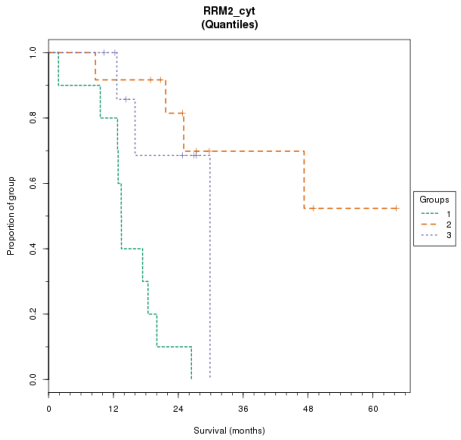
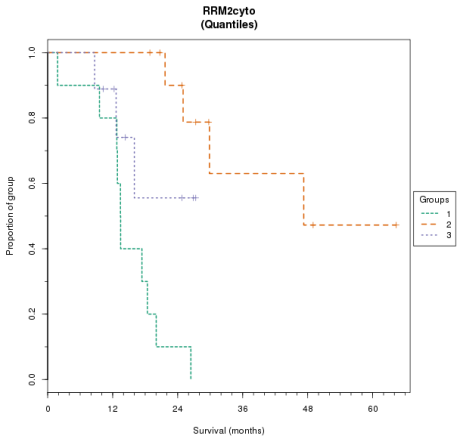
Median values			Combat data		
					
p = 0.0002			**p = 0.0001**		
Group (n=15)	**Expression**	**Median value**	**Group (n=15)**	**Expression**	**Median value**
1	105.96 - 1728.37	1376.24	1	105.96 - 1913.75	1467.90
2	2073.89 - 3445.94	2577.97	2	2037.72 - 3445.94	2577.97
3	3495.84 - 12468.20	4283.77	3	3717.51 - 12468.20	4653.50

Figure 3.30 Kaplan-Meier (tertile) plots of colorectal primary cancer TMA RRM2 cytoplasm expression showing proportion of group against disease free survival in months (median values versus combat data all patients who received 5-FU)

There are no statistically significant results for the cohort of colorectal cancer primary patients who did not receive chemotherapy in this TMA set.

It is very interesting that RRM2 cytoplasm expression in the liver secondary tumour TMA sets are all statistically insignificant.

The graph below was generated using the statistical function in excel and shows the change in cytoplasm RRM2 expression from primary tumour to secondary. Expression not only changes from high to low but also low to high.

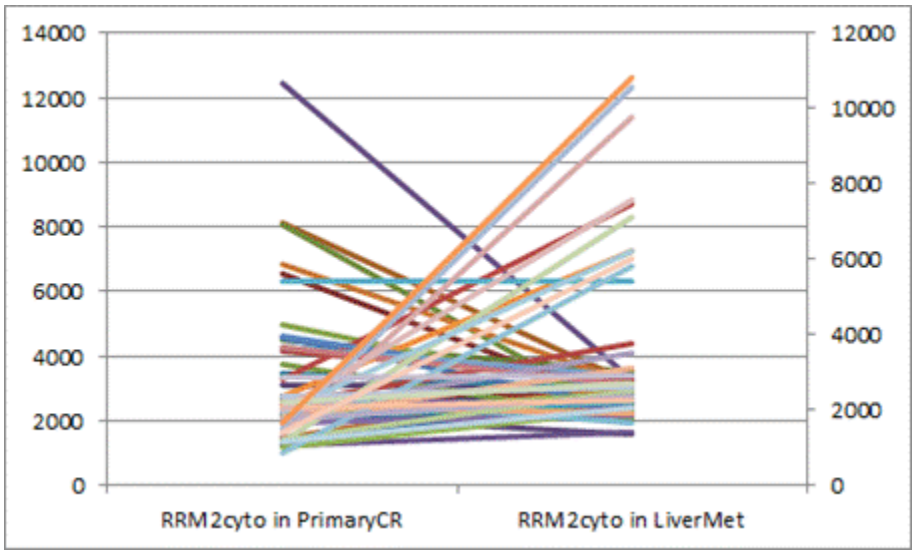


Figure 3.31 Graph showing the change in RRM2 cytoplasm expression in colorectal cancer primary tumour on the left hand side to the matched liver metastatic tumour on the right

3.9 Discussion

Quality

A quality management system was implemented for this research study to examine whether doing so actually contributed to or altered the final results in any way. The quality results are arguably more important than the biological results, as they could potentially impact on every single MSc and PhD project, as well as all academic research. Below is a list of quality steps and outcomes:

- The TMA map was reconciled against the blocks and slides. One error was found during this check and the block and slide discarded.
- TMA sections were checked by Professor Harrison to exclude any that contained no cancer. If these had been included in the final analysis it is highly probable that this would have generated different results.
- TMA sections were cut and stored at what is believed to be the optimum conditions. This was checked and validated by storing sections at different temperatures and comparing.
- An attempt was made to validate the antibodies available to researchers. This highlighted the variation in quality of antibodies and showed that it is a game of chance when purchasing antibodies.
- AQUA analysis was performed using two separate pieces of equipment. The results from both were quality checked and it was found the results were consistent across both machines.
- All data collected and analysed was QC checked. Some transcription errors were found and these were corrected before the final QA audit. Has these errors been missed it is possible that the results generated would be different.
- The final QA audit reconciled results against source data to ensure that what was reported was what was generated. The only reason this was error free was because of the QC checking implemented. If no quality checks were undertaken the final biological results may have turned out completely different.

The final results of this project would have been considerably different if the QA/QA checks were not implemented. If the TMA map had not been reconciled then the wrong results would have been recorded which alone may not have impacted hugely on the results. However, add to this the fact that the TMA cores were checked by an expert and non-cancerous cores excluded, the results would have been very different if these had been included.

It is of paramount importance to QC check any results wherever possible and this was included in this project. If the errors found had not been corrected then the results would have been different.

If time and word count had permitted, it would have been extremely enlightening to conduct two arms to this project in parallel – implementing quality in one arm and omitting quality in the other. This would entail double the results presented however, it would be conclusive evidence that quality should be included in all research, no matter the size of the project.

Pancreatic cancer

Pancreatic cancer is one of the most challenging solid tumours to treat, mainly due to the fact that less than 20% of patients present with resectable tumours as a direct consequence of the fact that pancreatic cancer is symptom-free in the early stages or symptoms mimic other diseases.[53 96] Pancreatic cancer patients with unresectable, metastatic disease have an average survival time of 2-6 months. Because time is so limited in this patient cohort there is an urgent need to stratify patients to tailored therapy that will increase survival time. It is also important that these patients, who have such a limited life span, are not subjected to chemotherapeutic treatments of which the side-effect:benefit ratio renders it pointless.

As described in chapter 2, automated quantitative analysis system AQUA was utilised to quantify potential prognostic and predictive biomarkers. These were then analysed both singly and in combination.

Univariate analysis

1. The complete patient set was evaluated for OS and DFS to see if any single protein expression showed a prognostic value. Low CDA expression and low RRM1 expression were associated with longer DFS in the complete set of patients with borderline statistical significance (p=0.06). There was nothing significant in OS for this group.
2. The group of patients who received gemcitabine was evaluated for OS and DFS. Low RRM1 expression corresponded with longer overall and disease free survival (p=0.02 and p=0.04 respectively).
3. The group of patients who didn't receive any chemotherapy was evaluated for OS and DFS. This was to check whether the results generated by the group that received gemcitabine were specific to this group or whether this was an effect being seen across both groups. In the group of patients who received no chemotherapy, there was nothing significant for OS and DFS.

Multivariate analysis

DCK is responsible for the phosphorylation of gemcitabine to gemcitabine monophosphate. RRM1 and RRM2 are responsible for catalysing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides. Thus it is reasonable to hypothesise that increased survival time could be associated with high DCK and low RRM1/ RRM2 expression.
Lecca et al described an algorithmic model of gemcitabine activity involving DCK, RRM1 and RRM2.[128] A final equation was created using DCK, RRM1 and RRM2 - DCK/(RRM1*RRM2) – and this equation was used as a means of multivariate analysis.

1. The complete patient set was analysed for OS and DFS; there was no difference in survival between the different expression groups.
2. The group of patients who received gemcitabine was evaluated for OS and DFS; a high Lecca value was linked to increased OS and DFS ($p = 0.007$ and $p = 0.07$ respectively). Taken at face value, this would suggest that a high Lecca value could potentially be a predictive biomarker for gemcitabine.
3. The group of patients who didn't receive chemotherapy was analysed for OS and DFS. This group showed the same stratification of expression groups as patients who received gemcitabine. That is, the high expression group showed longer OS and DFS. Analysing this group of patients has shown that increased OS and DFS is not specifically linked to the gemcitabine group, but also seen in the group that didn't receive chemotherapy.

Comparing the gemcitabine group with the no chemotherapy group; at 30 months (high expression group) 26% of patients who received no chemotherapy and 43% of patients who received gemcitabine were progression free.
24% of patients who received no chemotherapy (high expression group) were still alive at 48 months and 25% of patients who received gemcitabine (high expression group) were still alive at 72 months. Both the gemcitabine and untreated groups show the same high expression trend however, the gemcitabine group showed longer survival in the high expression group.
In the low expression groups, both sets of patients had the same amount of time to disease progression, 14 months. This would indicate that the Lecca formula may be useful in randomising patients not to receive gemcitabine and that offering the drug to patients with a high Lecca score could potentially prolong survival.

hENT1 is considered an independent predictor of gemcitabine resistance as it is one of the main proteins involved in transport into the cell. Transport of gemcitabine across the membrane is essential however there are several transporters capable of this - SLC28 transporters are sodium-dependent concentrative nucleoside transporters (CNTs), whereas the SLC29 transporters are sodium-independent equilibrative nucleoside transporters (ENTs) and consist of SLC29A1, SLC28A1, and SLC28A3.[130] It is important to take into account the subcellular location of hENT1 – if it is not localised to the cell membrane then no matter how great the amount of hENT1, it would not facilitate the transport of gemcitabine across the cell membrane if retained within the cell. [131] hENT1 expression effects on survival in this project were not conclusive.

It could be argued that biomarker research should be conducted via prospective clinical trials. Using retrospective FFPE samples is a relatively simple way to perform rapid analyses. The downside to this is the patient numbers tend to be low when the research is conducted as a single site, and the lack of robust negative controls or disregard of untreated group results could lead to incorrect interpretation of data.[135] [136] One way to counteract this would be to conduct biomarker research via a multi-centre study: this would ensure that the study is powered adequately and ensure that a control or placebo group is in place.
One clinical trial compared gemcitabine with FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin) therapy in patients with metastatic pancreatic cancer, with response rates of 9.4% and 31.6% respectively. [132] [133]
These response rates reinforce the necessity of further research into drug resistance. They also highlight the fact that there must be some other underlying reason why patients do not respond to gemcitabine therapy; it is not as straightforward as using protein biomarkers to predict response. The mechanisms involved in gemcitabine metabolism have been documented for many years, yet there remains no definitive answer mainly due to conflicting results and inadequate sample size. [95]

More research is required into the mechanisms of drug resistance, for example the signalling pathways which regulate the cell cycle and apoptosis. One research group found that the level of MAPK/ERK pathway activation correlates with sensitivity to gemcitabine.[134] These pathways may be targets for drugs which could assist in gemcitabine sensitivity however, targeting one pathway can result in disruption to another pathway rendering the subject of drug resistance both dynamic and very complex.
Other researchers have looked at tumour associated macrophages (TAMs) and found that infiltration of peripheral M2 macrophages were associated with disease progression and

resistance to gemcitabine, so it is possible that cancer cells are only part of the determinant of ultimate outcome after therapy. [135] [136]

Colorectal cancer

The colorectal cancer TMA set showed that the cytoplasmic expression of DPYD was statistically significant. Low expression was linked to longer overall survival in the cohort of all patients and longer overall and disease free survival in the Dukes C/D cohort of patients who received 5-FU. There were no significant findings in the cohort of patients who did not receive chemotherapy.

What is really interesting about these results is the fact that the AQUA expression levels were extremely low. If the staining had been done as routine clinical IHC then all the sections would have been scored negative. Perhaps this result should be held up as a reason to routinely stain using IF for AQUA analysis instead of IHC?

The colorectal primary cancer TMA set results are also interesting. It would not be inconceivable to expect this patient cohort to have a similar result to the colorectal cancer Dukes C/D cohort. Instead the cytoplasmic expression of RRM2 is statistically significant. High expression was linked to longer overall survival in the cohort of all patients and longer overall and disease free survival in the patient group that received 5-FU. There were no significant findings in the group of patients who did not receive chemotherapy.

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